Table IV—Intracerebral 6-Hydroxydopamine Effect on Norepinephrine and Dopamine Concentration in Mice Brains

Days after Injection[a]	Norepinephrine			Dopamine		
	Vehicle[b], μg/g ± *SE*	6-Hydroxy-dopamine, μg/g ± *SE*	Change, %	Vehicle, μg/g ± *SE*	6-Hydroxy-dopamine, μg/g ± *SE*	Change, %
14	0.308 ± 0.02	0.126 ± 0.03[c]	−59.09	0.287 ± 0.03	0.082 ± 0.03[c]	−71.43
42	0.336 ± 0.01	0.135 ± 0.03[c]	−59.82	0.298 ± 0.02	0.082 ± 0.02[c]	−72.48

[a] The mice brains (groups of six) were removed and assayed for catecholamine content 14 and 42 days following the intracerebral water or 6-hydroxydopamine injection (100 μg). [b] Aqueous ascorbic acid solution (0.5%) with pH adjusted to 5.5. [c] Compared to the appropriate vehicle, $p < 0.05$.

chlorpromazine possesses adrenolytic and antihistaminic qualities (14). Chlorpromazine may partially reverse the cobalt response through a central α-receptor blockade and/or peripheral antihistaminic activity.

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Antifertility and Antiproteolytic Activity of Activated *N*-Carbobenzoxy Amino Acid Esters

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Abstract ☐ *N*-Carbobenzoxy L-phenylalanine, glycine, L-leucine, and L-proline derivatives, their vinyl esters, and their 1,2-dibromoethyl esters were tested for antifertility activity in mice. Intraperitoneal administration reduced the pregnancy percentage and the number of fetuses per litter. Intravaginal administration reduced the pregnancy percentage significantly, with *N*-carbobenzoxyglycine vinyl ester, *N*-carbobenzoxyglycine-1,2-dibromoethyl ester, *N*-carbobenzoxy-L-leucine-1,2-dibromoethyl ester, and *N*-carbobenzoxy-L-proline-1,2-dibromoethyl ester producing 100% inhibition at 10 mg/kg/day. Sperm enzyme hydrolysis of the nonspecific substrate azocasein was inhibited significantly by certain *N*-carbobenzoxy amino acid esters *in vitro*. Specific substrate *N*-benzoyl-L-arginine ethyl ester hydrolysis was also inhibited. Compounds that inhibited *N*-benzoyl-L-arginine ethyl ester hydrolysis also demonstrated *in vivo* intravaginal antifertility activity.

Keyphrases ☐ Carbobenzoxy amino acid esters—contraceptive activity, antiproteolytic activity, mice ☐ Contraceptives, potential—activated *N*-carbobenzoxy amino acid esters, mice ☐ Enzyme activity—effect of activated *N*-carbobenzoxy amino acid esters on sperm proteolysis, mice ☐ Sperm—enzymes, effect of *N*-carbobenzoxy amino acid esters, mice

Recently, numerous activated *N*-protected amino acid esters were synthesized as possible latent proteolytic inhibitors (1, 2). These agents resemble known trypsin and chymotrypsin inhibitors. Certain members of the series effectively inhibited *in vivo* cathepsin activity and *in vitro* chymotrypsin activity (3). Since proteolytic enzyme inhibitors block sperm acrosin activity, capacitation, and fertility, the effects of this series on reproduction were investigated.

EXPERIMENTAL

Chemistry—*N*-Carbobenzoxy-L-phenylalanine (I), *N*-carbobenzoxyglycine (II), *N*-carbobenzoxy-L-leucine (III), and *N*-carbobenzoxy-L-proline (IV) were prepared by the standard procedure (4) (Table I). The *N*-carbobenzoxy amino acids were converted to the corresponding vinyl esters V, VI, VII, and VIII by refluxing with vinyl acetate in the presence of palladium chloride and were purified by column chromatography. The vinyl esters were treated with bromine in chloroform to give the 1,2-dibromo esters IX, X, XI, and XII, which were purified by fractional crystallization or column chromatography (1, 2). *N*-α-Tosyl-L-lysylchloromethyl ketone (XIII), tosyl-L-phenylalanylchloromethyl ketone (XIV), carbobenzoxy-L-phenylalanine chloromethyl ketone (XV), 17-ethinyl estradiol (XVI), and diethylstilbestrol (XVII) were purchased[1].

Antifertility Screens—For 28 days, virgin CF_1 female mice (~28 g), which had been isolated for 4 weeks, were administered test compounds suspended in 1% carboxymethylcellulose at 10 mg/kg/day (0.2 ml) ip. 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 a week to assure fertility. On gestation Days 17–21, the females were sacrificed;

[1] Sigma Chemical Co. or Cyclo Chemicals.

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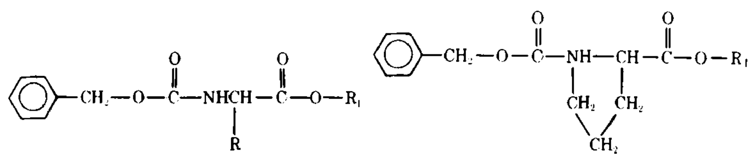

Table I—Structures of the *N*-Carbobenzoxy-Activated Amino Acids

	Compound	R	R_1	Melting Point	Preparation Method (Reference)
I	*N*-Carbobenzoxy-L-phenylalanine	$—CH_2—C_6H_5$	—H	86–89°	4
II	*N*-Carbobenzoxyglycine	—H	—H	117–119.5°	4
III	*N*-Carbobenzoxy-L-leucine	$—CH_2CH(CH_3)_2$	—	Oil	4
V	*N*-Carbobenzoxy-L-phenylalanine vinyl ester	$—CH_2—C_6H_5$	$—CH{=}CH_2$	Oil	1
VI	*N*-Carbobenzoxyglycine vinyl ester	—H	$—CH{=}CH_2$	Oil	2
VII	*N*-Carbobenzoxy-L-leucine vinyl ester	$—CH_2CH(CH_3)_2$	$—CH{=}CH_2$	Oil	2
IX	*N*-Carbobenzoxy-L-phenylalanine-1,2-dibromoethyl ester	$—CH_2—C_6H_5$	$—CHBr\text{-}CH_2Br$	Oil	1
X	*N*-Carbobenzoxyglycine-1,2-dibromoethyl ester	—H	$—CHBr—CH_2Br$	Oil	2
XI	*N*-Carbobenzoxy-L-leucine-1,2-dibromoethyl ester	$—CH_2CH(CH_3)_2$	$—CHBr—CH_2Br$	60–61°	2
IV	*N*-Carbobenzoxy-L-proline		—H	Oil	4
VIII	*N*-Carbobenzoxy-L-proline vinyl ester		$—CH{=}CH_2$	Oil	2
XII	*N*-Carbobenzoxy-L-proline-1,2-dibromoethyl ester		$—CHBr—CH_2Br$	Oil	2

Table II—Antifertility Screen of Activated Esters of *N*-Carbobenzoxy Amino Acids in CF_1 Females at 10 mg/kg/day

	Intraperitoneal Administration				Intravaginal Administration			
Compound	*n*	Percent Pregnant	Number of Viable Fetuses per Litter	Number of Intrauterine Deaths per Litter	*n*	Percent Pregnant	Number of Viable Fetuses per Litter	Number of Intrauterine Deaths per Litter
I	7	28	7.5	0.00	8	25	6.5	0.0
II	7	57	8.5	0.00	8	25	9.5	0.0
III	7	71	7.5	0.40	8	12	7.0	3.0
IV	8	38	9.6	0.33	8	25	8.0	0.0
V	10	100	10.7	0.10	8	25	7.0	0.0
VI	8	12	11.0	0.00	8	0	0.0	0.0
VII	8	62	11.8	0.00	8	25	7.0	0.0
VIII	6	43	7.3	0.00	8	25	10.5	0.0
IX	6	67	8.0	0.16	8	25	8.5	0.5
X	8	38	10.3	0.25	8	0	0.0	0.0
XI	8	38	12.4	0.13	8	0	0.0	0.0
XII	8	31	12.4	0.13	8	0	0.0	0.0
XIII	7	71	8.6	0.20	7	0	0.0	0.0
XIV	—	—	—	—	8	0	0.0	0.0
XV	8	88	11.6	0.14	8	38	9.0	0.0
XVI	8	0	0.0	0.0	—	—	—	—
XVII	8	0	0.0	0.0	—	—	—	—
1% Carboxymethylcellulose	8	12.0 ± 3	0.48	—	—	—	—	—
40% Lactose	—	—	—	—	8	100	11.0	0.70
6% Carboxymethylcellulose	—	—	—	—	8	75	8.8	0.66

the viable fetuses, intrauterine deaths, and implantation sites were tabulated (5, 6). Viable fetuses were examined for teratogenic effects as outlined previously (7).

For intravaginal testing, the compounds were homogenized in 40% lactose or 6% carboxymethylcellulose and administered at 10 mg/kg/day (0.03–0.05 ml) for 28 days using 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. On Day 10 and all subsequent days, females were exposed to males for 1 hr and the previously described protocol was followed. Dose-response curves for intravaginal administration were obtained over the 0.5–10-mg/kg/day range. Activated *N*-protected amino acid ester toxicity (LD_{50}) in CF_1 male mice (~30 g) was determined (8).

Enzymatic Studies—The acrosin enzyme was isolated from the epididymides of four Sprague-Dawley rats (~250 g) or 10 CF_1 mice (~30 g). The epididymides were sliced in 0.25 *M* sucrose–0.001 *M* disodium (ethylenedinitrilo)tetraacetate, pH 6.5. The sperm suspension was filtered through two sterile cheesecloth layers to remove debris, centrifuged at 600×*g* for 15 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 vortexed for 30 sec and centrifuged 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° (9, 10).

Acrosin proteolytic enzymatic activity was determined (11). 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. Absorbance of the trichloroacetic acid-soluble peptides produced by azocasein hydrolysis was measured at 366 nm. Enzymatic activity was calculated as the increase in absorbance of 0.730 unit/hr/mg of protein (11, 12).

N-Benzoyl-L-arginine ethyl ester hydrolysis was followed as described previously (12). Two milliliters of buffer, 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

Table III—Effects of *N*-Carbobenzoxy-Activated Esters at 1.5 μmoles on *In Vitro* Sperm Proteolytic Activity[a]

Compound	Percent Inhibition: Rat Azocasein Proteolytic Activity[b]	Percent Inhibition: Mouse *N*-Benzoyl-L-arginine Ethyl Ester Proteolytic Activity[c]	LD_{50}, mg/kg
I	93 ± 15	69 ± 5	250
II	63 ± 5	60 ± 1	380
III	68 ± 7	83 ± 7	>1000
IV	38 ± 4	77 ± 4	>1000
V	100 ± 1	57 ± 14	>2000
VI	96 ± 3	91 ± 2	500
VII	90 ± 14	68 ± 4	500
VIII	92 ± 2	79 ± 3	500
IX	85 ± 7	58 ± 3	74
X	100 ± 0	82 ± 1	148
XI	94 ± 2	85 ± 2	81
XII	94 ± 12	91 ± 1	225
XIII	100 ± 0	91 ± 1	—
XIV	100 ± 0	17 ± 2	75
XV	100 ± 0	35 ± 4	—

[a] All data in table have a $p = 0.001$ as calculated by the Student t test. [b] A 0.730-unit absorbance increase/hr/mg of protein. [c] A 0.328-unit absorbance increase/30 min/mg of protein.

were mixed, and the rise in absorbance at 253 nm was determined at 0 and 30 min. The control resulted in an absorbance increase of 0.328 unit/mg of protein (11, 12).

RESULTS

In the intraperitoneal antifertility screen, I, IV, VI, VIII, and X–XII produced greater than 50% pregnancy inhibition at 10 mg/kg/day compared to diethylstilbestrol and 17-ethinyl estradiol, which produced 100% pregnancy inhibition (Table II).

In the intravaginal antifertility screen, all tested compounds produced 75% or greater fertility inhibition. Compounds VI and X–XII caused 100% inhibition, as did the standard proteolytic inhibitors XIII and XIV. Dose–response curves were determined for VI, X, XI, and XIV at 0.5, 1, 5, and 10 mg/kg/day. Compounds VI, X, and XI caused 100% pregnancy inhibition at 1 mg/kg/day, whereas XIV required 10 mg/kg/day. No teratogenic effects were observed in the viable fetuses. Reabsorptions and intrauterine deaths were within normal limits (Table II).

Compounds I, V–VIII, and X–XV produced 90% rat acrosome proteolytic activity inhibition *in vitro*. Compounds III, VI, and X–XIII produced greater than 80% inhibition of *N*-benzoyl-L-arginine ethyl ester hydrolysis by mouse sperm acrosin *in vitro*; the remaining compounds produced greater than 50% inhibition (Table III).

DISCUSSION

Prior to fertilization, the sperm must undergo capacitation. This process involves sperm hydrolytic enzyme activation to permit penetration of the cervical mucus and external oval membranes as well as labilization of the external sperm membrane. Spermal acrosin (EC 3.4.21.10) has been isolated from various mammalian sperm (13) and apparently is responsible for zona pellucida penetration.

Acrosin, an endopeptidase that cleaves the carboxyl bond of arginyl and lysyl derivatives (13–15), can be inhibited by trypsin inhibitors (16–20). Endogenous acrosin inhibitors have been isolated from seminal plasma (21, 22), cervix uteri secretions (12), and pancreatic tissue (11). Normally, these inhibitors are removed as the sperm moves through the female reproductive tract, allowing the proteolytic enzyme to become active.

Screening demonstrated that the activated *N*-carbobenzoxy amino acid esters possessed antifertility activity in mice. Since the acrosome contains a number of proteolytic enzymes, any of which may hydrolyze azocasein, azocasein hydrolysis inhibition by the test compounds, chymotrypsin inhibitors (XIV and XV), and the trypsin inhibitor (XIII) was not surprising. Compounds VI and X–XII, which caused 100% pregnancy inhibition, also inhibited 80% of the hydrolysis of *N*-benzoyl-L-arginine ethyl ester, a substrate specific for acrosin and trypsin. Carbobenzoxy-activated esters also inhibit cathepsin (3). Thus, in addition to inhibiting sperm acrosin or trypsin, they may be reducing other proteolytic acrosomal enzymes. Since these *N*-carbobenzoxy-activated esters of L-proline, L-leucine, and glycine have no apparent teratogenic or toxic effects, they may have contraceptive potential.

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