with computer-generated random numbers.

Having obtained MSDs for a random sample of $B$ subsets (not all of which need be distinct), we determine the number A among them (counting "repeats" as often as they occur) for which the MSD is at least as small as that of the subset of observed active compounds. As before the ratio

$$\hat{p} = A/B \tag{17}$$

is formed. Unlike the case of exhaustive enumeration, however, this ratio is not the true $p$ value but simply an estimate of it (hence the caret).

The fraction of all subsets with an MSD at least as small as that of the "active" one is in fact the true but unknown $p$. Mathematically, the count $A$ has a binomial distribution with probability parameter $p$ and sample size parameter $B$. From this distribution confidence limits for $p$, incorporating the uncertainty due to sampling, can be obtained. Approximate confidence bounds, at the 95% level of confidence, are given by[22]

$$\hat{p} \pm 1.96[\hat{p}(1-\hat{p})/B]^{1/2} \tag{18}$$

Of course, the significance of the observed clustering must be evaluated in any given case by recalling that the true value of $p$, estimated by the bounds of eq 18, can be thought of as an ordinary significance probability.

**Registry No.** 7, 52372-93-7; 8, 52372-97-1; 9, 52373-02-1; 10, 52373-03-2; 11, 52373-04-3; 12, 52373-05-4; 13, 52372-95-9; 14, 52372-98-2; 15, 52373-06-5; 16, 52373-07-6; 17, 52373-08-7; 18, 52372-99-3; 19, 52373-09-8; 20, 52373-00-9; 21, 52373-01-0; 22, 52373-10-1; 23, 52372-94-8; 24, 52372-96-0; 26, 52373-12-3; 27, 61481-83-2; 28, 72738-92-2; 29, 53251-06-2; 30, 72739-01-6; 31, 61462-73-5; 32, 53222-10-9; 33, 58658-21-2; 34, 72738-97-7; 35, 72739-02-7; 36, 61417-04-7; 37, 72738-91-1; 38, 53222-12-1; 39, 59748-51-5; 40, 61417-03-6; 41, 61417-08-1; 42, 90-45-9; 43, 61417-13-8; 44, 53478-38-9; 45, 61417-05-8; 46, 64894-90-2; 47, 53478-39-0; 48, 53221-79-7; 49, 59748-95-7; 50, 57164-73-5; 51, 64894-94-6; 52, 58682-45-4; 53, 61417-07-0; 54, 53222-14-3; 55, 581-29-3; 56, 58658-24-5; 57, 66147-73-7; 58, 72738-90-0; MAO, 9001-66-5.

(22) Reference 18, p 246.

# Synthesis and Inhibition of Human Acrosin and Trypsin and Acute Toxicity of Aryl 4-Guanidinobenzoates

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The aryl 4-guanidinobenzoate, 4′-nitrophenyl 4-guanidinobenzoate (NPGB), is a potent inhibitor of sperm acrosin, an enzyme with an essential function in the fertilization process. NPGB prevents fertilization in a number of animal species and is a good lead compound for the development of contraceptive agents. In order to assess the efficacy of other aryl 4-guanidinobenzoates as acrosin inhibitors, 24 of these compounds were synthesized. Their inhibitory activity toward human acrosin was determined and compared with their activity toward human pancreatic trypsin in order to assess whether inhibitor sensitivity differed between these similar enzymes. Nine of the inhibitors were synthesized from phenols approved by the FDA for therapeutic use. The acute toxicity of these inhibitors in mice was determined and compared to that of nonoxynol-9, the most commonly used active ingredient in today's vaginal contraceptive preparations. All of the compounds proved to be potent inhibitors of human acrosin although 3 orders of magnitude difference were observed between the most and least effective inhibitors. Little specificity was present in regard to their inhibition of acrosin and trypsin. All the aryl 4-guanidinobenzoates synthesized from FDA-approved phenols were less toxic than nonoxynol-9, and it is concluded that these 4-guanidinobenzoates are of interest for further development and testing as nonhormonal contraceptive agents.

Acrosin, a serine proteinase with trypsin-like specificity and inhibitor sensitivity,[1] is associated with the sperm acrosome and has an essential function in the fertilization process. Spermatozoa appear to require acrosin for one or more of the following: (1) the sperm acrosome reaction, (2) sperm binding to the zona pellucida, the innermost of three layers surrounding the ovum during fertilization, and (3) lysis of a passage for the spermatozoon through the zona pellucida.[2] Thus, in the absence of acrosin, spermatozoa are unable to penetrate and fuse with the egg. Indeed, the addition of both naturally occurring and synthetic acrosin inhibitors to spermatozoa has been shown to prevent fertilization both in vitro and in vivo in the rabbit, rodent, and primate.[3]

Acrosin is specific to spermatozoa and makes an excellent target for the development of new, nonhormonal contraceptives (i.e., acrosin inhibitors). Several such inhibitors such as $N^{\alpha}$-tosyl-L-lysine chloromethyl ketone (TLCK),[3] 4′-nitrophenyl 4-guanidinobenzoate (NPGB),[3] $N$-carbobenzoxy amino acid esters,[4,5] and sterol sulfates[6-8]

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have already been shown to have vaginal contraceptive properties in animal species. NPGB is of particular interest because it is a highly active acrosin inhibitor and it is as potent upon vaginal application to primates as is a presently marketed vaginal contraceptive (nonoxynol-9, a detergent with spermicidal properties) but at much lower concentrations.[9] NPGB also prevents the fertilizing capacity of mouse spermatozoa in vitro[10] and rabbit spermatozoa in vivo;[11-13] other aryl 4-guanidinobenzoates inhibit the in vitro penetrability of human spermatozoa.[14] Thus, NPGB represents a good model compound upon which to base the synthesis of additional acrosin inhibitors whose high activity against acrosin and low toxicity may lend themselves as effective contraceptive agents.

Many aryl guanidinobenzoates have been evaluated as serine proteinase inhibitors. Among the many guanidino esters that have been tested and that exhibit some of the best inhibitory activity are the aryl 3- and 4-guanidinobenzoates,[17-21] aryl guanidinonaphthoates,[22] aryl guanidinotetrahydronaphthoates,[22] and recently the guanidinoaryl benzoates.[23] Among the most potent of the inhibitors is 4'-nitrophenyl 4-guanidinobenzoate (NPGB), which is also used as an active-site titrant for trypsin. An analogue of NPGB in which the 4-nitrophenol moiety has been replaced with a fluorescein derivative has also been synthesized and found to be an excellent active-site titrant for serine proteinases.[24]

Another aryl 4-guanidinobenzoate, 4'-methylumbelliferyl 4-guanidinobenzoate (MUGB), is as effective as NPGB in preventing the in vitro fertilization of mouse gametes but is considerably less toxic.[10] It is likely that the toxicity of the aryl 4-guanidinobenzoates is, at least in part, determined by the toxicity of the phenolic leaving group. In regard to NPGB and MUGB, 4-nitrophenol is more toxic than 4-methylumbelliferone, an FDA-approved therapeutic agent. As with trypsin,[15] NPGB most probably interacts with human acrosin to form a stable acyl–enzyme intermediate, with the concomitant release of 4-nitrophenol. The questionable toxicity of 4-nitrophenol poses the potential problem of its release in the vagina. Therefore, it was the first objective to synthesize a series of aryl 4-guanidinobenzoates, composed in part of phenols that themselves are currently used as therapeutic agents (e.g., acetaminophen and methylparaben) or are included in some vaginal douche preparations (i.e. 8-quinolinol).[16]

It was hoped that these aryl 4-guanidinobenzoates would have high acrosin inhibitory activity and have low toxicity. To evaluate the latter, as the second objective, acute toxicity studies were conducted in mice and comparisons were made with nonoxynol-9, a sperm-immobilizing agent and the active ingredient in most presently marketed vaginal contraceptives. As the third objective, a number of similar ortho-, meta-, and para-substituted aryl 4-guanidinobenzoates were also synthesized in order to explore substituent effects and determine the best structural requirements for acrosin inhibitory activity. A large variety of para substituents in the phenolic part of these esters were chosen to relate activity with either or both electronic and steric effects of such substituents. Several similar meta- and ortho-substituted analogues were also included to compare to the para analogues. These compounds were also tested against human trypsin to ascertain any specificity of inhibition. Since the ultimate goal is to develop clinically applicable agents and since the acrosin sensitivity to inhibitors varies among species,[1] human sources were used for the isolation of these enzymes.

## Results and Discussion

All the aryl 4-guanidinobenzoates were highly potent and effective inhibitors of both human acrosin and human trypsin with $I_{50}$ values ranging between $5 \times 10^{-7}$ to $7 \times 10^{-10}$ M (Table I). The 4'-carbomethoxyphenyl ester was the most active of the inhibitors with $I_{50}$ values of $6.7 \times 10^{-10}$ and $1.5 \times 10^{-9}$ M, repsectively, whereas the 2-isopropyl-5-methylphenyl and the 2-(aminocarbonyl)phenyl esters were the least effective toward both acrosin and trypsin. The 4-CN-, 4-Cl-, and 4-Br-substituted phenyl esters were also among the more potent of the acrosin inhibitors with $I_{50}$ values in the range of ca. $1-4 \times 10^{-9}$ M. Previous experiments with NPGB[25] and determinations with MUGB that were performed with the synthesized aryl 4-guanidinobenzoates gave $I_{50}$ values for these inhibitors with human acrosin of 4.0 and $5.5 \times 10^{-9}$ M, respectively.

In general, the 4-substituted phenyl esters were more effective acrosin inhibitors than the corresponding 2- and 3-substituted phenyl esters. However, on the basis of the observed acrosin inhibitory activity of the aryl 4-guanidinobenzoates, no direct correlation could be made between the electronegativity of the substituents and their $I_{50}$ values. Several phenyl esters containing electron-withdrawing substituents (e.g., 4-$CF_3$ and 4-$CO_2CH_2CH_3$) were equally or even less effective in inhibiting acrosin than those containing electron-donating groups (e.g., 4-$CH_3$ and 4-$CH(CH_3)_2$). However, the present data suggest the importance of steric effects in determining relative inhibitory activities with the 4-alkyl-substituted phenyl esters. There is a gradual decrease in inhibitory potency observed as the size of the substituent is increased from methyl to *tert*-butyl. Similarly, as one goes from the 4-carbomethoxy-

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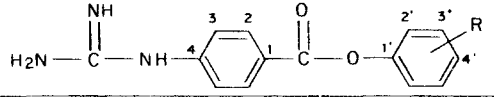
**Table I.** Human Acrosin and Trypsin Inhibition and Acute Toxicities of Aryl 4-Guanidinobenzoates



| compd | R | salt | method[a] | mp,[b] °C | recrystn solvent | yield, % | formula[c] | inhibition: $10^9 I_{50}$, M[d] human[e] acrosin | inhibition: $10^9 I_{50}$, M[d] human[f] trypsin | $LD_{50}$,[g] mg/kg |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | H[h] | | A | 340 dec | ethanol | 34 | $C_{14}H_{13}N_3O_2$ | 5.6 | 23.4 | |
| **2** | 2′-$OCH_3$ | | A | >500 | ethanol | 27 | $C_{15}H_{15}N_3O_3$ | 27.3 | 87.6 | |
| **3** | 2′-$CO_2CH_3$ | | A | 320 dec | ethanol | 70 | $C_{16}H_{15}N_3O_4$ | 12.9 | 65.0 | 750 (478–1178) |
| **4** | 2′-$CONH_2$ | HCl | B | 237–239 dec | methanol–ether | 58 | $C_{15}H_{15}ClN_4O_3$ | 300.0 | 300.0 | >1000[k] |
| **5** | 3′-$OCH_3$ | | A | 220 dec | chloroform | 16 | $C_{15}H_{15}N_3O_3$ | 17.6 | 16.5 | |
| **6** | 3′-$CF_3$ | | A | >500 dec | benzene | 42 | $C_{15}H_{12}F_3N_3O_2$ | 38.5 | 92.2 | |
| **7** | 3′-CN | HCl | B | 188.5–193.5 dec | ethanol | 45 | $C_{15}H_{13}ClN_4O_2$ | 7.2 | 3.8 | |
| **8** | 3′-$CO_2CH_3$ | HCl | B | 192–196 dec | ethanol | 56 | $C_{16}H_{16}ClN_3O_4$ | 5.4 | 27.1 | |
| **9** | 4′-$CH_3$[i] | | A | >500 dec | acetone | 48 | $C_{15}H_{15}N_3O_2$ | 3.3 | 19.5 | |
| **10** | 4′-$CH(CH_3)_2$ | | A | >500 dec | chloroform | 40 | $C_{17}H_{19}N_3O_2$ | 6.6 | 17.2 | |
| **11** | 4′-$C(CH_3)_3$ | | A | >500 dec | chloroform | 58 | $C_{18}H_{21}N_3O_2$ | 28.5 | 17.1 | |
| **12** | 4′-Cl[i] | 4-$CH_3C_6H_4SO_3H$ | B | 230–233 | methanol–ether | 36 | $C_{21}H_{20}ClN_3O_5S$ | 3.0 | 8.2 | |
| **13** | 4′-Br | 4-$CH_3C_6H_4SO_3H$ | B | 239.5–241.5 | methanol–ether | 73 | $C_{21}H_{20}BrN_3O_5S$ | 3.5 | 3.9 | |
| **14** | 4′-$OCH_3$ | | A | >500 | ethanol | 54 | $C_{15}H_{15}N_3O_3$ | 16.3 | 19.0 | |
| **14a** | 4′-$OCH_3$ | 4-$CH_3C_6H_4SO_3H$ | B | 216–222 | chloroform | 35 | $C_{22}H_{23}N_3O_6S$ | | | |
| **15** | 4′-$O(CH_2)_3CH_3$ | | A | >500 | chloroform | 71 | $C_{18}H_{21}N_3O_3$ | 11.4 | 22.3 | |
| **15a** | 4′-$O(CH_2)_3CH_3$ | 4-$CH_3C_6H_4SO_3H$ | B | 211–213 dec | | 31 | $C_{25}H_{29}N_3O_6S$ | | | |
| **16** | 4′-$NHCOCH_3$ | HCl | B | 251–254 | ethanol–hexane | 44 | $C_{16}H_{17}ClN_4O_3$ | 8.0 | 23.7 | 850 (691–1045) |
| **17** | 4′-$CF_3$ | | A | 275 dec | ethanol–pet. ether | 61 | $C_{15}H_{13}F_3N_3O_2$ | 33.0 | 16.4 | |
| **18** | 4′-CN[e] | 4-$CH_3C_6H_4SO_3H$ | B | 198–202 dec | ethanol | 23 | $C_{22}H_{20}N_4O_5S$ | 1.20 | 1.6 | |
| **19** | 4′-$CO_2CH_3$[i] | HCl | C | 255–257 dec | ethanol–hexane | 81 | $C_{16}H_{16}ClN_3O_4$ | 0.67 | 1.5 | 235 (205–269) |
| **20** | 4′-$CO_2C_2H_5$[j] | HCl | C | 207–210 dec | ethanol–hexane | 70 | $C_{17}H_{18}ClN_3O_4$ | 9.3 | 1.6 | 300 (234–384) |
| **21** | 2′-$OCH_3$, 4′-$CH_2CH{=}CH_2$ | | A | >500 dec | ethanol | 85 | $C_{18}H_9N_3O_3$ | 13.7 | 8.4 | 870 (762–994) |
| **22** | 2′-$CH(CH_3)_2$, 5′-$CH_3$ | 4-$CH_3C_6H_4SO_3H$ | B | 239–242 dec | methanol–ether | 26 | $C_{25}H_{29}N_3O_5S$ | 300.0 | 500.0 | >2000[k] |
| **23** | 3′-OH, 4′-$(CH_2)_5CH_3$ | | A | 250 dec | acetone | 69 | $C_{20}H_{25}N_3O_3$ | 99.7 | 18.5 | 620 (518–743) |
| **24** | 2′-N=CHCH=CH-3′ (8-quinolyl) | HCl | C | 245–249 dec | methanol–ether | 29 | $C_{17}H_{15}ClN_4O_2$ | 19.8 | 27.7 | 240 (218–264) |

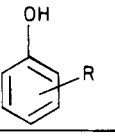
[a] General methods are described in the Experimental Section. [b] The melting points of these compounds was frequently diffuse and many of the above values represent decomposition points; mp's >500 °C dec mean that decomposition without the formation of a meniscus was observed. [c] All microanalyses were carried out by Micro Tech Laboratories, Skokie, IL, and are accurate to within ±0.4% of the calculated values. [d] Values represent mean values of duplicate determinations. [e] Control activity 11.3 μmol $min^{-1}$ $mg^{-1}$. [f] Control activity 7.3 μmol $min^{-1}$ $mg^{-1}$. [g] All compounds were suspended in 5% gum acacia; 5% gum acacia (control) had no effect on the mice; 95% confidence limits were calculated for all values according to the method of Litchfield and Wilcoxon.[33] [h] Reported as the nitrate (ref 17). [i] Reference 18. [j] Reported as the sulfate.[19] [k] The highest dose that could be administered ip due to the high viscosity of the gum acacia suspension. No deaths were observed at this dose level.

to 4-carbethoxyphenyl substituents there is a marked decrease in activity. On the other hand, the more hydrophilic 4-methoxyphenyl and 4-butoxyphenyl esters are approximately equal in potency. The observed differences could be the result of a combination of unfavorable steric and/or hydrophobic interactions. There also appears to be no absolute size requirement for the 4-substituted phenyl esters, since the 4-isopropyl or 4-*tert*-butyl group did not exhibit any large differences in inhibitory potency as compared to several of the others such as the 4-methoxy and 4-trifluoromethyl. The 2-(aminocarbonyl)phenyl ester showed a surprisingly sharp decrease in activity ($I_{50} = 3 \times 10^{-7}$ M), being ca. 450 times less potent than the 4-carbomethoxyphenyl derivative against both acrosin and trypsin. This could possibly be indicative of a limited hydrophobic binding area within the active site that is not favorable for hydrogen bonding or dipole–dipole interactions with the 2-aminocarbonyl group.

In terms of specificity, none of the aryl 4-guanidinobenzoates exhibited any significant degree of selectivity in inhibitory activity toward acrosin or trypsin. This suggests that the topography in and around the active site of human acrosin may be analogous to that of human trypsin. In general, the majority of inhibitors tended to be equally or slightly more active against acrosin than trypsin. It should be noted, however, that the relative value of substrate to Michaelis constant ($S/K_m$) used to assay trypsin was somewhat higher than that used to assay acrosin. This may offer an explanation for the somewhat greater efficacy of these pesudoirreversible inhibitors against acrosin as compared to trypsin but only if there were a reversible component of inhibition. The possible occurrence of such a reversible component is presently being investigated. The largest differences in reactivity observed were those with the 4-methylphenyl ester, which was ca. 6-fold more active against acrosin than trypsin, while the carbethoxyphenyl and the 3-hydroxy-4-hexylphenyl esters were about 5–6 times more effective as trypsin inhibitors. In addition, the 3-carbomethoxyphenyl ester proved to be about 5-fold more potent in inhibiting acrosin than trypsin.

This lack of specificity is not surprising since acrosin is classified as a trypsin-like enzyme and resembles trypsin in many respects, such as inhibitor and substrate specificity, although distinct differences exist in several of their physical and catalytic properties. The most notable physical differences are in their apparent molecular weights and isoelectric points. Human acrosin has a molecular weight of 70 000 and a p*I* of 9.0 whereas human trypsin has a molecular weight of 23 000 and a p*I* of 8.2.[26] In addition, recent evidence suggests that acrosin, unlike trypsin, may be comprised of multiple subunits and/or active sites.[27,28] The majority of alkyl and aryl guanidinobenzoates that have been tested as antiproteolytic agents are also relatively nonspecific in terms of inhibitory activity toward various proteinases. To date, the only known inhibitors of human acrosin without trypsin inhibitory activity are certain monosaccharide derivatives[29] and 3-hydroxy-5-sterol sulfates.[7] Further kinetic studies are warranted in order to determine the reversibility of these aryl 4-guanidinobenzoates with human acrosin and human trypsin. Quantification of rates of enzyme acylation by these agents may be helpful in more precisely identifying structural requirements for specific enzyme inhibitors.

**Table II.** Food and Drug Administration Approved Phenols




| substit R | name | pharmacological activity |
|---|---|---|
| 2-$CONH_2$ | salicylamide | analgesic |
| 2-$CO_2CH_3$ | methyl salicylate | counterirritant<br>flavoring agent |
| 4-$NHCOCH_3$ | acetaminophen | analgesic<br>antipyretic |
| 4-$CO_2CH_2CH_3$ | ethylparaben | preservative |
| 4-$CO_2CH_3$ | methylparaben | preservative |
| 2-$OCH_3$, 4-$CH_2CH{=}CH_2$ | eugenol | dental obtundent<br>topical anesthetic |
| 3-OH, 4-$(CH_2)_5CH_3$ | hexylresorcinol | antiseptic |
| 2-$CH(CH_3)_2$, 5-$CH_3$ | thymol | antibacterial<br>antifungal |
| 2,3-(N=CHCH=CH) | 8-hydroxyquinoline | antimicrobial |

A number of the aryl 4-guanidinobenzoates incorporated phenols that are presently used clinically (Table II). The $LD_{50}$'s of these compounds ranged from 230 to >2000 mg/kg (Table I). Under exactly the same experimental conditions, nonoxynol-9, the most widely used spermicide on the market, was more toxic than any of these compounds, possessing an $LD_{50}$ of 190 mg/kg. In this test system, NPGB gave an $LD_{50}$ of 500 mg/kg, while the commercially available 4-methylumbelliferyl ester (MUGB) was found to have an $LD_{50}$ of >2000 mg/kg. Higher concentrations of MUGB could not be used due to the high viscosity of the gum acacia suspension which prevented accurate intraperitoneal injection. This same problem arose with levels of the 2-(aminocarbonyl)phenyl compound above 1000 mg/kg. The higher $LD_{50}$ value obtained for NPGB in the present study (500 mg/kg) as compared to a value of 180 mg/kg reported for NPGB previously[10] is probably due to its suspension in gum acacia rather than in $Me_2SO$ as was used before. This may be due to higher effective concentrations of the substances when in solution as opposed to in suspension. Alternatively, the higher toxicity of NPGB when $Me_2SO$ was the vehicle could represent an interaction between the two substances.

The majority of inhibitors were less toxic than NPGB. These results support the premise that the toxicity of aryl 4-guanidinobenzoates can be at least partially attributed to the nature of the leaving group. Additionally, the data show that the aryl 4-guanidinobenzoates are equally as or less toxic than agents now in clinical use.[30] The acute toxicity studies were done for comparisons only. Further evaluations of the subacute/chronic toxicity, vaginal irritation, and mutagenic and teratologic properties of the compounds need to be made before they can find clinical use.

Recently, the literature on the use of enzyme inhibitors as antifertility agents has been reviewed.[31] Too few data are available at present to compare the relative merit of the various enzyme inhibitors that have been tested.

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However, the high acrosin inhibitory activity exhibited by a number of the aryl 4-guanidinobenzoates as well as their low toxicity make them good candidates for further development and testing as contraceptive agents. Their low molecular weight and relative hydrophobicity should allow for the penetration of the plasma and acrosomal membranes of the spermatozoa so that they can contact acrosin and inactivate the spermatozoa. Additionally, these properties of the aryl 4-guanidinobenzoates should allow their distribution throughout the vagina and their rapid entry into the seminal coagulum that forms immediately upon ejaculation, often adjacent to the cervix. Many spermatozoa are trapped in this coagulum and enter the cervix immediately after the coagulum liquifies. Nonoxynol-9 is a surfactant and may have difficulty passing into the coagulum so that many spermatozoa escape immobilization by this detergent. Thus, the aryl 4-guanidinobenzoates should have the advantage over nonoxynol-9 from a clinical standpoint in being able to rapidly contact the spermatozoa.

The clinical applicability of these agents as contraceptives is, of course, subject to empirical verification. Vaginal contraceptive studies have already been performed in rabbits with the compounds that contained phenols approved by the FDA for human use.[32] All compounds were tested at 0.1 mg/mL (2 mL/rabbit vagina). The 2′-carboxamidophenyl, 2′-isopropyl-5′-methylphenyl, and the 3′-hydroxy-4′-hexylphenyl derivatives that possessed the lowest acrosin inhibitory activity were also the poorest antifertility agents, producing mean percent fertilizations of 94.6%, 55.7%, and 40.0%, respectively. The controls possessed a mean fertilization of 82.3%. The 4′-carbethoxyphenyl, 4′-acetamidophenyl, 2′-carbomethoxyphenyl, 4′-carbomethoxyphenyl, 2′-methoxy-4′-allylphenyl and the 8′-quinolyl derivatives were effective contraceptives, reducing the fertilization rates to 0.3%, 8.3%, 10.1%, 15.1%, 16.3%, and 25.7%, respectively. Nonoxynol-9, under exactly the same conditions but at 10- and 100-fold higher concentrations, also showed an antifertility effect: 32.4% fertilization at 1 mg/mL and 16.3% at 10 mg/mL. However, even at these increased dose levels, the contraceptive efficacy of nonoxynol-9 was not higher than that of most of the inhibitors and was more variable than that of the most active aryl 4-guanidinobenzoates. Thus, these rabbit data together with the present results, indicate that several aryl 4-guanidinobenzoates are more potent and less toxic than nonoxynol-9, justifying further study of these compounds for clinical purposes.

## Experimental Section

Melting points were determined on a Mel-Temp block and are uncorrected. $^1$H NMR spectra were obtained in $Me_2SO$-$d_6$ with a Varian T-60A spectrometer equipped with a Nicolet Instrument Corp. TT-7 Fourier transform accessory. Chemical shifts ($\delta$) are reported in parts per million (ppm) downfield from $(CH_3)_4Si$ as internal standard. The abbreviations s and m refer to singlet and multiplet, respectively. A Gilford Model 250 recording spectrophotometer was used for spectrophotometric assays of acrosin and trypsin.

Methyl and ethyl 4-hydroxybenzoates, 8-hydroxyquinoline, salicylamide, 4-guanidinobenzoic acid hydrochloride, *N*-$\alpha$-benzoyl-L-arginine ethyl ester (BAEE), *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (Hepes), tris(hydroxymethyl)aminomethane hydrochloride (Tris), and dimethyl sulfoxide ($Me_2SO$) were purchased from Sigma Chemical Co. (St. Louis, MO). Dicyclohexylcarbodiimide (DCC), *p*-cresol, 4-isopropylphenol, 4-*tert*-butylphenol, 4-chlorophenol, 4-bromophenol, 3- and 4-hydroxybenzonitrile, 2- and 4-methoxyphenol, 4-butoxyphenol, 3- and 4-trifluoromethylphenol, and methyl 3-hydroxybenzoate were obtained from Aldrich Chemical Co. (Milwaukee, WI). Nonoxynol-9 was kindly donated by Ortho Pharmaceutical (Raritan, NJ).

DCC was melted prior to use, weighed out, and used immediately. Anhydrous 4-toluenesulfonic acid was prepared from the monohydrate by several azeotropic distillations with benzene just prior to use. Pyridine was kept over fresh NaOH pellets (24 h), then refluxed over $CaH_2$ (3 h), distilled, and stored over molecular sieves (4 Å). Dimethylformamide (DMF) was dried over molecular sieves (4 Å, for 24 h), and was distilled through a fractionating column over ninhydrin (1 torr) and then stored over molecular sieves (4 Å). Human semen was obtained from Dr. S. Leto (Washington Fertility Study Center, Washington, DC). The semen was frozen and shipped in liquid nitrogen and stored at −70 °C. Postmortem human pancreas were obtained from the Department of Pathology, University of Illinois at Chicago, Health Sciences Center, and was stored at −20 °C prior to use.

**Aryl 4-Guanidinobenzoates.** All the esters were prepared by the DCC-assisted condensation of the requisite phenol with 4-guanidinobenzoic acid by a modification of the technique of Holmberg and Hansen.[33]

**Method A. Isolation of the Guanidines as Free Bases.** 4-Guanidinobenzoic acid hydrochloride (10.00 g, 0.046 mol), the phenol, (0.14–0.21 mol), and anhydrous 4-toluenesulfonic acid (4.6–18.0 mmol) were suspended in anhydrous pyridine (30 mL) at 0 °C. After 15 min, a solution of DCC (0.14–0.21 mol) in dry DMF (30 mL) was added at once and the mixture stirred at room temperature for 2–3 days. Acetic acid (15.0 mL) was added to destroy excess DCC. After the mixture was stirred for 1 h, solids were filtered off and washed with DMF (50 mL). The ratio of dicyclohexylurea (DCU) to the guanidino ester hydrochloride and/or toluenesulfonate was determined from $^1$H NMR spectra (in $Me_2SO$-$d_6$) by integrating the aliphatic protons of DCU (two broad multiplets centered at 1.24 and 1.63 ppm) and the aromatic protons (approximately between 7.35 and 8.30 ppm). To avoid counting exchangeable protons in this ratio, $D_2O$ was added to exchange water and guanidine NH protons (7.00–8.25 ppm). If the salt was a 4-toluenesulfonate, the $CH_3$ resonance (s at 2.31 ppm) could be used as a monitor. In some experiments the initial solid that was filtered off consisted of 80–90% of DCU which was recrystallized from 2-propanol (mp 235–38 °C, lit. mp 232–234 °C). Once it was established that the majority of the initial product was DCU, the guanidine was isolated from the filtrate.

To obtain the base, ice (150 g) and 5% ice-cold NaOH (200 mL) were added to the mother liquor. If a precipitate appeared, it was filtered and identified. If the guanidino ester did not precipitate at this point, the mixture was mixed with methylene chloride (100 mL). In many instances, the product then appeared at the methylene chloride–aqueous interface. The solid was filtered and washed with water until the washings were neutral. The alkaline biphasic filtrate was separated into an organic and an aqueous layer. The aqueous phase was extracted several times with methylene chloride, and all organic extracts were combined, washed with water (3 × 100 mL), dried ($Na_2SO_4$) and evaporated in vacuo, and examined for additional product. The base was recrystallized to constant melting point (see Table I).

**Method B. Isolation of Guanidinium Salts.** The filtrate from DCU (and salts) was diluted with ice–water (150-mL total volume), acidified with 5 N HCl (pH 1) and layered onto methylene chloride (100 mL). Usually, the guanidinium salt crystallized at the interface. The solid was filtered, washed with water (50 mL), and methylene chloride (50 mL), examined by $^1$H NMR for its identity, and purified (see Table I).

**Method C.** In several experiments, the initial precipitate that was filtered off consisted of a mixture of DCU, together with the majority of the salt of the expected guanidino ester. One of the best methods of separating the salt from DCU was accomplished by continuous extraction (Sohxlet) of DCU into chloroform. This separation was followed by periodic examination of the $^1$H NMR spectrum of the solid material in the Sohxlet thimble. Once the salt was free from DCU, it was purified by recrystallization. The

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original filtrate from the mixture of DCU and the salt was examined for additional product by either method A or method B.

**Enzyme Inhibition Studies.** Human acrosin was isolated from frozen human semen according to the procedure of Anderson et al.[25] Acrosin-containing extracts were obtained by acid extraction of spermatozoa, and acrosin was further purified by the sequential use of Sephadex G-150, CM-cellulose, and DEAE-cellulose chromatography. Human trypsin was isolated and purified from frozen human pancreas as previously described.[25] Trypsinogen was isolated from human pancreas by acid extraction and ammonium sulfate fractionation. After activation of the trypsinogen to trypsin, purification was achieved by Sephadex G-50 and SP-Sephadex chromatography. Protein concentration was measured by reaction with fluorescamine.[34]

Acrosin and trypsin activity was measured spectrophotometrically by following the rate of increase in absorbance at 253 nm with BAEE as substrate.[27] The acrosin assay mixtures contained the following final concentrations of reagents: 50 mM Tris-HCl (pH 8.0), 0.05 mM BAEE, 0.5–2.0 μg of enzyme protein, and various concentrations of inhibitor in a total volume of 1.0 mL. Reaction mixtures for trypsin assays consisted of 50 mM Tris-HCl (pH 8.0), 0.2 mM BAEE, and 0.3–1.0 μg of enzyme protein in a total volume of 1.0 mL. Different protein concentrations were used to achieve the same esterolytic activity (see legend, Table I) since different enzyme preparations possessed somewhat different specific activities. Each determination was done in duplicate. All inhibitors (1 mM) were dissolved in $Me_2SO$ and diluted with water to their final concentrations. Inhibitors were preincubated with either acrosin or trypsin at pH 8.0 (50 mM Tris-HCl) for 5 min at 25 °C to allow temperature equilibration prior to initiating the reaction with substrate (BAEE). It was assumed that an increase of 1.15 absorbance units was equivalent to the hydrolysis of 1.0 μmol of BAEE.[35]

All reaction rates were expressed as μmol of BAEE hydrolyzed $min^{-1}$ (mg of protein)$^{-1}$. The $I_{50}$ values (the amount of inhibitor that reduced the enzyme activity by 50%) were calculated from plots of percent control activity vs. log inhibitor concentration under the experimental conditions employed. Plots were subjected to linear regression analysis: all correlation coefficients were greater than 0.97.

**Toxicological Studies.** $LD_{50}$ values (that dose of the compounds that will produce death in 50% of the animals within 24 h after acute treatment) were determined for the aryl 4-guanidinobenzoates that were synthesized from FDA-approved phenols (Table I). All compounds were suspended in 5% gum acacia and administered via intraperitoneal injection. Mature, male Swiss Webster albino mice with a minimum body weight of 25 g were used, and the volume injected was 0.1 mL/10 g of body weight. The $LD_{50}$ values were determined from dose–response curves according to the method of Litchfield and Wilcoxon.[36] Similar studies were performed with nonoxynol-9 for comparative purposes.

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