A NITROSATED ARGININE DERIVATIVE, A POWERFUL MUTAGEN.

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SUMMARY: An arginine derivative, benzoyl-L-arginine-amide, when nitrosated, showed a powerful mutagenic action on E. coli and Salmonella typhimurium. The active principle was identified to be 4-benzoylamido-4-carboxamido-n(N-nitroso)butylcyanamide. The mutagenic activity of the new compound was more than 30 times higher than that of N-methyl-N'-nitro-N-nitrosoguanidine at neutral pH.

Arginine is considered to be structurally similar to N-methyl-N'-nitroguanidine (MNG) in such a sense as to contain guanido group in its molecule. MNG is easily converted by nitrosation reaction under acidic condition to the corresponding nitroso compound, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)[1]. MNNG has been well known to be a powerful mutagen[2,3] and was shown by Sugimura et al. to be capable of inducing cancer in high frequency in glandular stomach of rodents as well as in stomach of dogs by oral administration[4.5.6]. Thus, it seems reasonable to expect mutagenic and carcinogenic activities in nitrosated products of arginine and its enviromental derivatives. From this point of view, we first nitrosated various arginine derivatives and searched for their mutagenic activities. During this study we found a very powerful mutagen in nitrosated products of benzoyl-L-arginineamide (BAA). This paper describes the preparation, structure identification and mutagenic activity of this new compound.

MATERIALS AND METHODS:

E. coli K12 strain, B9601 (tryptophan amber auxotroph), and Salmonella typhimurium strains, TA1535, TA1536, TA1537, and TA1538 (his^- gal^- bio^- uvrB deep rough mutants) were used for the mutagenic assay. The latter strains were kindly given by Dr. Bruce N. Ames, Biochemistry Department, University of California, Berkeley.

BAA hydrochloride monohydrate was purchased from Sigma Chem. Co. The other reagents were commercially obtained.

BAA was nitrosated by dropwise addition of sodium nitrite solution in 1:3 molar ratio in 1.3 N nitric acid at 0^oC for 2 hrs (see legend to photo 1). The reaction mixture was then neutralized with sodium carbonate, adjusted to a definite volume, sterilized by Millipore (HA type) filtration, serially diluted and subjected to the spot test for mutagenicity assay.

Log phase cells of the E. coli strain grown in M9 medium was washed twice and resuspended with M9 buffer to give its OD_{660} in 1.0 and 0.1 ml aliquot was spread on semi enriched medium plate[7] 1 hr before assay. 0.1 ml of overnight culture of the Salmonella strains in nutrient broth was plated on minimal agar according to the method of Ames[8], 1 hr before assay.

The test solutions were spotted respectively on the plates and incubated at 37^oC for 2 to 3 days.

RESULTS AND DISCUSSION

As shown in Fig. 1 (A)(B), the nitrosated BAA solution produced a circle of colonies around the spot on B9601 and on TA1535. The spot of control solution lacking either BAA or nitrite in the reaction mixture scarcely formed

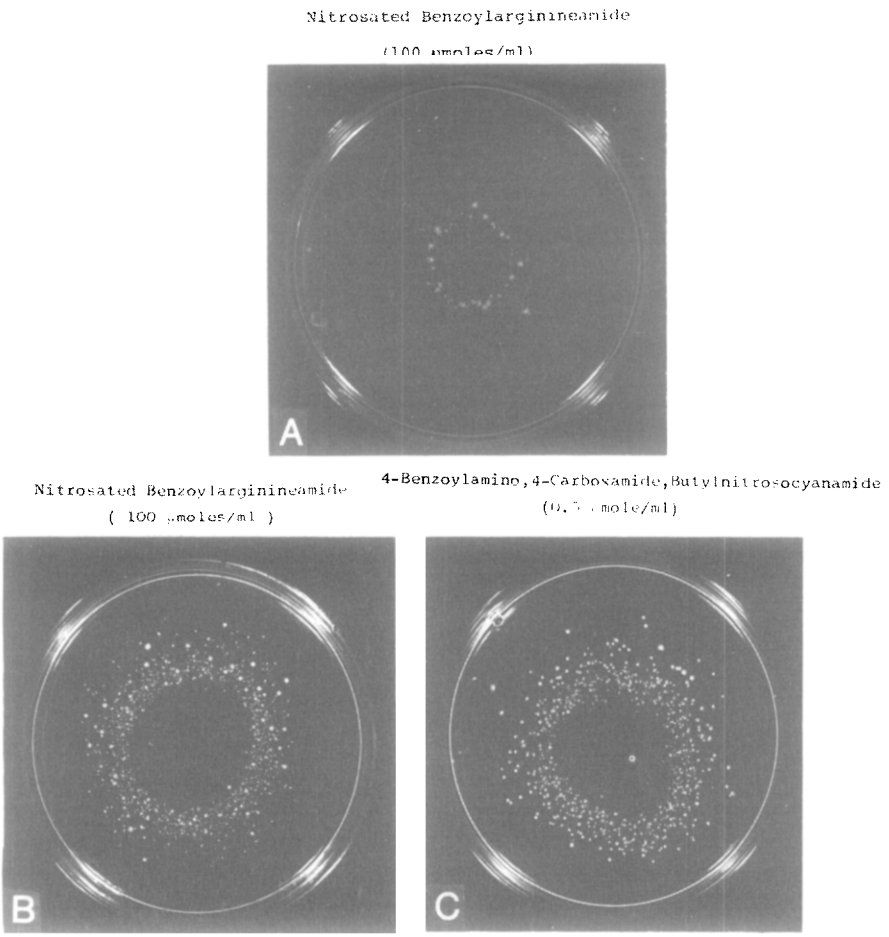
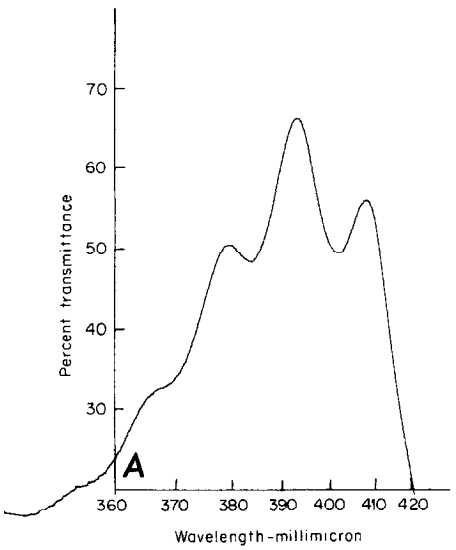


Fig. 1. The standard reaction mixture for mutagenic test contains 66.4 mg (0.2 mmole) of BAA hydrochloride monohydrate which was dissolved in 0.1 ml of prewarmed dimethylformamide, 0.2 ml of distilled water, 0.06 ml of concentrated nitric acid and 0.2 ml (0.63 mmole) of sodium nitrite aqueous solution(230 mg/ml). The reaction mixture was neutralized with sodium carbonate after 2 hrs incubation at 0°C, adjusted to 2 ml with phosphate buffer (pH 7.2), sterilized by Millipore (HA type) filtration, spotted on the plates of B9601 (A) and TA1535 (B). The active principle of nitrosated BAA, 4-benzoylamido-4-carboxamido-n(N-nitroso)-butylcyanamide was dissolved in ethanol, diluted with sterilized phosphate buffer (pH 7.2) to a concentration of 0.5 μmole/ml of 5% ethanol and spotted on TA1535 (C). The plates were incubated at 37°C for 2 days.

colonies, indicating clearly that the phenomenon observed is not due to BAA or nitrous acid itself. So far as tested, the colonies randomly picked up from both plates were all try^+ and his^+ respectively. Thus, the circle

formation of colonies around the spot is concluded to be revertants caused by a mutagenic principle in nitrosated BAA solution. The mutagenic test of serially diluted fractions revealed that the nitrosated product in the original reaction mixture was effective by 64 fold dilution on B9601, while on TA1535, by 5120 fold dilution.

For the purification and identification of the active principle, 3 mmoles of BAA was nitrosated in the same manner as already described, and the reaction mixture was extracted with 25 ml each of cold ethylacetate 3 times, immediately after neutralisation. Ethylacetate extracts were then washed with saturated sodium chloride solution 3 times (each 10 ml) to remove dimethylformamide which was used to dissolve BAA in agueous solution. After dryness over sodium sulfate, ethylacetate solution was concentrated under vacuum. The concentrate, when developed on thin layer silica gel plate, gave a red spot of Rf 0.6 by spraying Griess reagent. Silica gel of the thin layer chromatogram of the concentrate was divided into two parts, a zone corresponding to Rf 0.5-0.7 and the others, scraped and extracted with small amount of absolute ethanol. The mutagenic activity was only seen in the extract of the former. The concentrate was applied to a column (5.5 x 1.3 cm) of kieselgel G (Merck, 10-40 u) and fractionated by eluting it with ethylacetate. The fractions showing Griess reagent positive spot on thin layer chromatogram with Rf 0.6 were collected and evaporated under vacuum. The remaining oily substance gradually converted to crystal form. This was recrystallized from ethylacetate and ligroin. The physical properties of



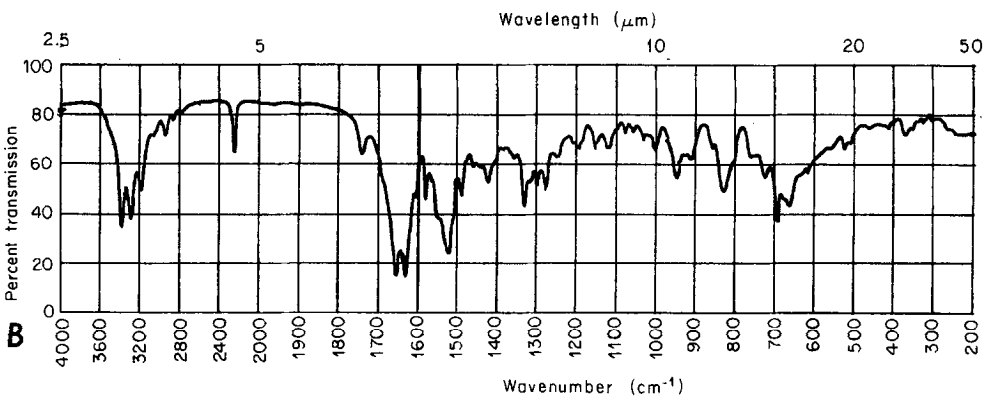


Fig. 2 (A). Absorption spectrum of 4-benzoylamido-4-carboxamido-n(N-nitroso)butylcyanamide (5 x 10^{-3}M) in ethanol.

(B). IR spectrum of 4-benzoylamido-4-carboxamido-n(N-nitroso)butylcyanamide (KBr).

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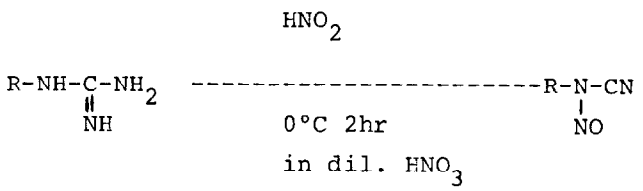
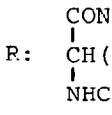
this compound was as follows. mp. 110°C (decomp); colorless needles; UV, λ_{max} (ε), at 409(71.6), 393(94.3), 379(61.1) and one shoulder at 367 nm in ethanol (Fig. 2 (A)); IR spectrum, typical CN band at 2240 cm^{-1} (Fig. 2 (B)); Elementary analysis, calculated for $C_{13}H_{15}O_3N_5$: C,53.97; H,5.23; N,24.21, Found: C,53.74; H,5.12; N.24.36.

Thus, the active principle was identified to be 4-benzoyl-amido-4-carboxamido-n(N-nitroso)butylcyanamide. Mass spectrum analysis also supported the structure. The yield of the pure compound was 132.3 mg (13.3%) in our conditions.





The mutagenic action of this new compound was demonstrated in Fig. 1 (C).

Next, the mutagenic activity of this compound was compared with that of MNNG by serially diluting the stock solution (0.5 μmole/ml of 5% ethanol) of the respective compound with phosphate buffer (pH 7.2) and spotting them on TA1535. Surprizingly, the activity of the new compound was still positive in 1024 fold dilution (488 pmoles/ml), while that of MNNG was by 32 fold dilution (15.6 nmoles/ml). This means the mutagenic activity of the new compound is more than 30 times higher than that of MNNG at neutral pH. This compound also showed slightly mutagenic effect on TA1538 but no activity on TA1536 and TA1537. On the other hand, MNNG was weakly mutagenic on TA1537 but not on TA1536 and TA1538. The results were summarized in Table 1. The strain TA1535 was developed as a tester strain for detecting base pair substitution mutagens and the strains TA1536, TA1537, and TA1538 for screening frame shift mutagens[8,9]

Table 1. Comparison of mutagenic activity between 4-benzoylamido-4-carboxamido-n(N-nitroso)butylcyanamide and N-methyl-N'-nitro-N-nitrosoguanidine on Salmonella typhimurium strains.

The original solution of both compounds (0.5 µmole/ml of 5% ethanol) was serially diluted with phosphate buffer (pH 7.2) and spotted on the plates of TA1535, TA1536, TA1537 and TA1538 respectively (see MATERIALS AND METHODS). +,- means revertant formation positive and negative. RNC is a tentative abbreviation of 4-benzoylamido-4-carboxamido-n(N-nitroso)butylcyanamide.

Tester Strain	Dilution 0.5 µmole/ml	x1	x2	x4	x8	x16	x32	x64	x128	x256	x512	x1024	x2048
TA1535	RNC	+	+	+	+	+	+	+	+	+	+	+	-
	MNNG	+	+	+	+	+	+	-					
TA1536	RNC	-											
	MNNG	-											
TA1537	RNC	-											
	MNNG	+	+	-									
TA1538	RNC	+	+	+	+	-							
	MNNG	-											

It is therefore likely that the mutagenicity of the new compound is predominantly due to base pair substitution. Detailed studies on the mutagenic action of this compound and carcinogenesis experiment are now in progress in our laboratory.

During the kinetical studies on the nitrosation reaction of alkylureas, alkylurethans and alkylguanidines, Mirvish observed that an unknown product was transiently formed during the nitrosation of methylguanidine.[10] This compound, though not isolated and identified, was suggested to be methylnitrosocyanamide by the spectrophotometrical evidence. Our present study seems to strongly support his observation. It is noteworthy that the N-nitrosocyanamide formation from guanidino compound proceeds in fairly good yield in aqueous solution in such a mild nitrosation condition as described in this paper.

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