Brit. J. Pharmacol. (1960), **15**, 243.

THE ANTIMICROBIAL PROPERTIES OF SOME α-AMINO-OXY-ACIDS, α-AMINO-OXY-HYDRAZIDES, ALKOXYAMINES, ALKOXYDIGUANIDES AND THEIR DERIVATIVES

BY

S. A. PRICE, P. MAMALIS, D. McHALE, AND J. GREEN

From the Walton Oaks Experimental Station, Vitamins Ltd., Tadworth, Surrey

A series of compounds containing an amino-oxy-group has been examined for antibacterial and antifungal activity. The amino-oxy-acids and the alkoxyamines showed little activity, but the hydrazides, notably the amino-oxydodecyl and the amino-oxytetradecyl compounds, had appreciable activity against *Staphylococcus aureus*. The alkoxydiguanides showed considerable bactericidal activity *in vitro* against Gram-positive and Gram-negative organisms and some activity against *Mycobacterium tuberculosis*. The most active compounds were those with a chain containing 10 to 14 carbon atoms, but the activity was considerably reduced in the presence of serum. They were also active against the few fungi tested. The most active compound, decyloxydiguanide, was moderately toxic when administered intraperitoneally to mice and no therapeutic activity could be demonstrated against an intraperitoneal injection of *Streptococcus pyogenes* administered 15 min. previously.

Canavanine, the *N*-amidino-derivative of an *O*-substituted hydroxylamine, is a powerful inhibitor of certain strains of *Neurospora* (Horowitz and Srb, 1948). Hydroxylamine itself shows slight antibacterial properties (Gray and Lambert, 1948), while certain alkoxyamines (I) and *N*-alkoxyguanidines (II) are active particularly against Gram-positive bacteria (Fuller and King, 1947).

$RONH_2$ (I) $RONH.C(:NH).NH_2$ (II) $RR'C(ONH_2).CO_2H$ (III)

$RONH.C(:NH).NH.C(:NH).NH_2$ (IV)

$RCH(ONH_2).CO.NH.NH_2$ (V)

$RCH(CO_2H).O.NH.C(:NH).NH.C(:NH).NH_2$ (VI)

The preparation of some compounds of types (I) and (III to VI) as potential antibacterials based on hydroxylamine has been described by McHale, Green, and Mamalis (1960) and by Mamalis, Green, and McHale (1960); the antibacterial properties of these compounds are now described. In order to facilitate comparisons with existing data, some of the alkoxyamines (I) of Fuller and King were re-examined. All compounds were tested *in vitro* against representative Gram-positive and Gram-negative organisms; the more active compounds were also screened against a wider range of bacteria including *Mycobacterium tuberculosis* and against some fungi. One compound was examined for its ability to protect mice against *Streptococcus pyogenes* infection.

METHODS

Antibacterial Studies in vitro.—All the compounds were screened against *Staphylococcus aureus* NCTC 4163 and *Escherichia coli* NCTC 8196 using a simple serial dilution test in nutrient broth containing Lab. Lemco 1%, Peptone (Evans) 1%, NaCl 0.5%, at *p*H 7.5. The compounds, in 0.12% aqueous solution adjusted to *p*H 6.8 to 7.0, were added in 5 ml. volumes to 5 ml. of double-strength nutrient broth to give 600 p.p.m. of the substance under test. Twofold dilutions were then made in single-strength broth. The tubes were autoclaved for 10 min. at 10 pounds/square inch pressure and, after cooling, inoculated with 0.02 ml. of a 6 hr. log-phase culture diluted 1 in 10^7. All tubes were incubated at 37° and the minimum inhibitory concentrations (M.I.C.) were observed after 48 hr. Sulphathiazole was included as a reference compound in all tests. Screening against *Streptococcus pyogenes* NCTC 8322, *Corynebacterium diphtheriae* NCTC 3989, *Eberthella typhosa* NCTC 160, *Klebsiella pneumoniae* PCI/602, and *Pseudomonas aeruginosa* NCTC 8203 was similarly carried out both in the absence and presence of 10% normal horse serum.

To determine whether the compounds were bactericidal or merely bacteriostatic, subcultures were

made from the tubes after 18 hr. incubation into large volumes of nutrient broth or on to agar plates. The bactericidal concentration was established as the minimal concentration from which living organisms could not be recovered.

For determination of activity against *Mycobacterium tuberculosis* H 37 Rv, twofold serial dilutions of the compounds in 3 ml. of Dubos and Davis Tween Albumin medium were infected with 0.02 ml. of a 10 day Dubos and Davis culture diluted 1 in 10 and incubated at 37° for 14 days. Isoniazid and *p*-aminosalicylic acid were included as standards for each batch of tests.

Antifungal Studies in vitro.—Antifungal activity was assessed using techniques essentially similar to those of Collier, Potter and Taylor (1955). Dequalinium chloride and chlorhexidine diacetate were used as reference compounds.

Antibacterial Studies in vivo.—Attempts were made to demonstrate systemic activity in mice by protection experiments using *Streptococcus pyogenes* (Richards). A culture of sufficient virulence was obtained after repeated passage through mice, and a 6 hr. log-phase culture used. An infecting dose of 0.2 ml. of a 1 in 10^6 dilution of such a culture (equivalent to 20 lethal doses) was administered intraperitoneally 15 min. before administration of the compound.

Results

Alkoxyamines, α-Amino-oxy-acids, and α-Amino-oxy-hydrazides

In confirmation of the observations of Fuller and King, we have found that the hydrochlorides of alkoxyamines (I) have only feeble antibacterial activity *in vitro* with M.I.C.s of the order 300 to 600 p.p.m. for both *Staph. aureus* and *E. coli* (sulphathiazole under the same conditions has an *in vitro* M.I.C. of 5 to 10 p.p.m.). Slightly greater activity was observed against *M. tuberculosis* (75 to 600 p.p.m.) as compared with the control compounds (0.125 to 0.5 p.p.m.). The following alkoxyamines (I) were examined: R = hydrogen (hydroxylamine), ethyl, n-propyl, isopropyl, n-butyl, n-pentyl, n-hexyl, heptyl, decyl, dodecyl, and benzyl.

The corresponding α-amino-oxy-acids (III) were slightly more active than the alkoxyamines (I) against *Staph. aureus* and *E. coli* (ca. 150 to 600 p.p.m.) while activity against *M. tuberculosis* (ca. 37.5 to 150 p.p.m.) was also somewhat greater. The following α-amino-oxy-acids (III) were examined: R = hydrogen, R′ = hydrogen, methyl, ethyl, n-propyl, isopropyl, n-butyl, n-pentyl, n-hexyl, heptyl, octyl, decyl, dodecyl, 4-cyclohexyl-butyl; R = R′ = methyl.

The possibility existed that the amino-oxy-acids might be amino-acid antagonists and this was investigated with α-amino-oxyisovaleric acid using *Leuconostoc mesenteroides* P. 60, an organism exacting towards valine, the analogous amino-acid. The growth-promoting effect of valine on a valine-deficient synthetic medium was found to be stimulated rather than inhibited by the amino-oxy-analogue, which in fact exerted a slight but significant sparing effect on valine utilization. In the absence of valine, the amino-oxy-compound was itself utilized to a limited extent, about 1,000 μg. being needed to replace 20 μg. valine.

Some α-amino-oxy-hydrazides (V; R = n-hexyl, heptyl, octyl, decyl, and dodecyl) were tested for antitubercular activity *in vitro* and shown to be rather more active (M.I.C.s 9 to 75 p.p.m.) than the analogous acids. All but two of the hydrazides showed little activity against *Staph. aureus* and *E. coli* (about 300 p.p.m.), the exceptions being (V; R = dodecyl and tetradecyl) which inhibited growth of *Staph. aureus* at 30 and 37.5 p.p.m. respectively.

Alkoxy- and Arylmethoxy-diguanides in vitro

While the alkoxyamines (I), the acids (III), and the hydrazides (V) showed little or no increase in activity against *Staph. aureus* and *E. coli* with increasing molecular weight, the same was not true of the alkoxydiguanides (IV). The hydrochlorides of a series of diguanides (IV; R = hydrogen, ethyl, n-butyl, isobutyl, n-pentyl, n-hexyl, heptyl, octyl, nonyl, decyl, undecyl, 2-methyldecyl, dodecyl, tetradecyl, and hexadecyl) were examined. The M.I.C.s for both the above two organisms were found to increase rapidly with increasing alkyl chain length, reaching a maximum (0.6 to 1.25 p.p.m.) at C_{10} to C_{12}. The highest members of the series showed reduced activity, perhaps associated with decreased aqueous solubility. The activity of these compounds against *M. tuberculosis* followed a similar pattern with a maximum effect at C_{10} to C_{12} (5 p.p.m.). Branching of the alkyl chain did not affect activity.

Introduction of a carboxyl group into the diguanides to give (VI; R = n-pentyl, n-hexyl, heptyl, octyl, and decyl) resulted in complete loss of *in vitro* activity.

Hydrochlorides of arylmethoxydiguanides were found, in general, to be less active than the alkoxydiguanides. Of the following which were tested [IV; R = benzyl, *p*-chloro-, *p*-bromo-, and 3,4-dichloro-benzyl, 6-chloro-1,3-benzodioxan-8-yl-methyl, 1- and 2-naphthylmethyl, and 1-bromo-2-naphthylmethyl], 2-naphthyl-methoxydiguanide hydrochloride was the most active against *Staph. aureus* (25 p.p.m.) and *E. coli* (12.5 p.p.m.) [cf. decyloxydiguanide dihydrochloride (1.25 p.p.m.) for both organisms].

TABLE I

MINIMAL EFFECTIVE CONCENTRATIONS (P.P.M.) OF SOME ACTIVE ALKOXYDIGUANIDES IN THE PRESENCE AND ABSENCE OF 10% SERUM

Column a: in broth. Column b: in broth + 10% normal horse serum.

	Alkoxydiguanides RONH.C(:NH).NH.C(:NH).NH_2								Reference Compounds					
	$R=C_8H_{17}$ No. 129		$R=C_{10}H_{21}$ No. 130		$R=C_{12}H_{25}$ No. 133		$R=C_{14}H_{29}$ No. 134		Sulphathiazole		Dequalinium Chloride	Chlorhexidine Diacetate
	a	b	a	b	a	b	a	b	a	b	a	a
Staph. aureus 4163 ..	10	50	0·6	37·5	1·5	75	3	75	5	600		
Strept. pyogenes 8322 ..	2·5	12·5	1·25	12·5	2·5	12·5	10	37·5	>300	>1,200		
C. diphtheriae 3989 ..	2·5	12·5	1·25	12·5	0·6	12·5	0·6	12·5	5	6·25		
E. coli 8196 ..	10	37·5	1·25	37·5	1·5	75	6·25	150	10	>600		
Kleb. pneumoniae 1/602 ..	12·5	40	5	30	5	40	100	150	300	400		
Sal. typhii 160 ..	12·5	30	2·5	40	2·5	50	5	>50	25	40		
Ps. aeruginosa 8203 ..	50	150	12·5	300	>200	>600	>200	>600	>600	>1,200		
Microsporon canis ..	20		2·5		2·5		20				<0·6	0·6
Candida albicans ..	>20		10		20		>20				<0·6	10
Trichophyton mentagrophytes ..	20		5		10		>20				<0·6	5
Epidermophyton floccosum ..	10		2·5		2·5		20				<0·6	5

The table shows the activity of the four most active alkoxydiguanides against a wider range of organisms, the least susceptible being *Ps. aeruginosa*. In the presence of 10% serum, the activities were diminished sharply, the M.I.C.s being increased from 5 to 50 fold. With each of the bacteria listed in the table, it was confirmed that the inhibition was bactericidal and not merely bacteriostatic.

The table also shows that antifungal and antibacterial activities run parallel, the peak effect being reached with the C_{10} and C_{12} compounds.

Toxicities

No adverse effects were noted with any of the four compounds in the table when neutralized suspensions (in 0.5 ml.) were injected subcutaneously into mice (19 to 21 g.) in doses up to 1,000 mg./kg. The mice were killed after 9 days and examined: it was found that the C_{10}, C_{12} and C_{14} compounds remained undissolved in the subcutaneous tissue. The more soluble acetate of decyloxyguanide (C_{10}) when injected subcutaneously at 1,000 mg./kg. produced no systemic effects, but severe necrosis developed at the site of the injection. The intraperitoneal toxicity of the latter compound was determined in mice; the LD50 (Miller and Tainter, 1944) was found to be 20 ± 2.5 mg./kg.

In vivo *Experiments with Diguanides*

The activity *in vivo* of decyloxydiguanide acetate (IV; R=decyl), the most active member of the series, was examined in groups of mice infected with *Strept. pyogenes* (Richards). Fifteen min. after intraperitoneal infection with 0.2 ml. of a 6 hr. log-phase culture, the compound was administered intraperitoneally at 10 mg./kg. The treated and control mice died within three days and no evidence for any prolongation of survival times was observed. Although no attempt has been made to isolate metabolic products, it would appear, therefore, that the compound

is excreted or metabolized too rapidly to show any protective effect; diguanides are known to react rapidly with ketones and keto-esters to form cyclic products.

We are indebted to Dr. J. H. Humphrey for the culture of *Streptococcus pyogenes* (Richards), to Dr. H. O. J. Collier and Dr. F. L. Rose for the samples of dequalinium, chloride and chlorhexidine diacetate respectively, and to Mr. K. J. Stevens for valuable technical assistance.

References

Collier, H. O. J., Potter, M. D., and Taylor, E. P. (1955). *Brit. J. Pharmacol.*, **10**, 343.

Fuller, A. T., and King, H. (1947). *J. chem. Soc.*, 963.

Gray, J. D. A., and Lambert, R. A. (1948). *Nature (Lond.)*, **162**, 733.

Horowitz, N. H., and Srb, A. M. (1948). *J. biol. Chem.*, **174**, 371.

McHale, D., Green, J., and Mamalis, P. (1960). *J. chem. Soc.*, 225.

Mamalis, P., Green, J., and McHale, D. (1960). Ibid., 229.

Miller, L. C., and Tainter, M. L. (1944). *Proc. Soc. exp. Biol. N.Y.*, **57**, 261.