Experimental Section

Melting points were detd with a Mel-Temp apparatus and are uncorrected. The optical rotations were detd in the solvents specified with a Rudolph Model 80 polarimeter. The uv spectra of all compds were detd in aq soln with a Cary Model 14 spectrophotometer and were as expected. The ir spectra were detd in pressed KBr discs with Perkin-Elmer Models 221-G, 521, and 621 spectrophotometers and were also normal. The pmr spectra were detd in DMSO-d_6 (TMS) with a Varian A-60A spectrometer; chemical shifts quoted in the case of multiplets are measured from the approximate center. Chromatographic analyses were carried out on tlc plates of silica gel H (Brinkmann). The spots were detected by uv light after spraying the plates with Ultraphor (WT, highly concd). Analytical samples were dried over P_2O_5 (0.07 mm) for 4–7 hr at 78 or 100°.

Preparation of D-Pentofuranosyl-8-azaadenines. A soln of the blocked D-pentofuranosyl chloride in dry C_6H_6 (*ca.* 20 ml/mmole) was added to a flask contg *N*-nonanoyl-8-azaadenine (1 equiv) and Molecular Sieve (1.8–2.5 g/mmole). The mixt was refluxed with stirring for 1 hr, addnl Molecular Sieve added (1.8–2.5 g/mmole), and heating contd for an addnl hr. Filtration of the cooled reaction mixt gave an insol material from which *N*-nonanoyl-8-azaadenine was recovered. A soln of the residue from evapn (*in vacuo*) of the filtrate in dry MeOH contg NaOMe (1.5 equiv) was refluxed for 0.5 hr, chilled, neutralized with AcOH, and evapd to dryness *in vacuo*. A soln of the residue in H_2O was washed with $CHCl_3$, concd, and chilled to give a cryst solid (except in the case of α-6, which was isolated as the picrate from the filtrate of the β-anomer). The analytical samples (**2**, α- and β-4, and β-6) were obtained by recrystn (see Table II). The *O*-benzyl groups of β- and α-6 were removed by hydrogenolysis at room temp in 1:1 MeOH–2-methoxyethanol at 50 psi for 2 hr using $PdCl_2$ catalyst (0.5 g/mmole).

9-β-D-Xylofuranosyl-8-azahypoxanthine (3-β-D-Xylofuranosyl)-*v*-triazolo[4,5-*d*]pyrimidin-7(6*H*)-one, **3**). To a soln of 9-β-D-xylofuranosyl-8-azaadenine (815 mg, 3.04 mmoles) in hot H_2O (30 ml) was added $NaNO_2$ (5.06 g, 73.4 mmoles) followed by glacial AcOH (5.06 ml). The reaction soln was stirred at room temp for 16 hr, diluted with H_2O (38 ml), and stirred for an addnl hr. Addn of 1.2 equiv of $Pb(OAC)_2$ followed by 10.3 ml of concd NH_4OH gave a white solid that was collected by filtration. A soln of this solid in 20% aq AcOH was treated with H_2S. The black PbS was removed by filtration and the filtrate evapd to dryness *in vacuo*. The residue crystd from 80% aq EtOH; yield, 334 mg. Addn of another 0.6 equiv of $Pb(OAc)_2$ to the filtrate followed by concd NH_4OH (5 ml) gave addnl material from which a second crop was obtd; yield, 78 mg (total yield 51%). The analytical sample was obtd by recrystn from abs EtOH; mp 174–176°. *Anal.* ($C_9H_{11}N_5O_5$) C, H, N.

2′-Deoxy-8-azainosine [3-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-*v*-triazolo[4,5-*d*]pyrimidine-7(6*H*)-one, β-5). Treatment of 2′-deoxy-8-azaadenosine (200 mg, 0.8 mmole) as described above gave the product (β-5), which crystd from EtOH; yield, 70 mg (28%). The analytical sample was obtd by recrystn from EtOH; mp 155–156°. *Anal.* ($C_9H_{11}N_5O_4 \cdot 0.1H_2O$) C, H, N.

3-(2-Deoxy-α-D-*erythro*-pentofuranosyl-*v*-triazolo[4,5-*d*]pyrimidin-7(6*H*)-one, α-5). In the same manner, 9-(2-deoxy-α-D-ribofuranosyl)-8-azaadenine (252 mg, 10 mmoles) gave the α-anomer of 2′-deoxy-8-azainosine, which crystd from 90% EtOH; yield 137 mg (54%); mp 143–144°. *Anal.* ($C_9H_{11}N_5O_4$) C, H, N.

Acknowledgment. The authors are indebted to Dr. W. C. Coburn, Jr., and members of the Molecular Spectroscopy Section of Southern Research Institute for spectral and microanalytical data, to Mrs. Martha Thorpe for her help on the interpretation of the pmr data, and to Dr. L. L. Bennett, Jr., and Mrs. Margaret H. Vail for the cytotoxicity data reported.

References

(1) P. W. Allan and L. L. Bennett, Jr., *Proc. Amer. Ass. Cancer Res.*, 11, 2 (1970).
(2) J. Davoll, *J. Chem. Soc.*, 1593 (1958).
(3) J. A. Montgomery, H. J. Thomas, and S. J. Clayton, *J. Heterocycl. Chem.*, 7, 215 (1970).
(4) W. W. Lee, A. P. Martinez, G. L. Tong, and L. Goodman, *Chem. Ind. (London)*, 2007 (1963).
(5) G. L. Tong, W. W. Lee, L. Goodman, and S. Frederiksen, *Arch. Biochem. Biophys.*, 12, 76 (1965).
(6) H. P. Schnebli, D. L. Hill, and L. L. Bennett, Jr., *J. Biol. Chem.*, 242, 1997 (1967).
(7) J. A. Montgomery, *Progr. Med. Chem.*, 7, 69 (1970).
(8) M. J. Robins and R. K. Robins, *J. Amer. Chem. Soc.*, 87, 4934 (1965).
(9) C. P. J. Glaudmans and H. G. Fletcher, *J. Org. Chem.*, 28, 3004 (1963).
(10) K. R. Darnall and L. B. Townsend, *J. Heterocycl. Chem.*, 3, 371 (1966).
(11) B. R. Baker, *Ciba Found. Symp. Chem. Biol. Purines*, 120 (1957).
(12) J. A. Montgomery and K. Hewson, *J. Med. Chem.*, 11, 48 (1968); 12, 498 (1969).
(13) L. L. Bennett, Jr., M. H. Vail, S. Chumley, and J. A. Montgomery, *Biochem. Pharmacol.*, 15, 1719 (1966).
(14) S. Minato, *J. Biochem.*, 64, 815 (1968).
(15) L. L. Bennett, Jr., H. P. Schnebli, M. H. Vail, P. W. Allan, and J. A. Montgomery, *Mol. Pharmacol.*, 2, 432 (1966).

Glutathione. 6. Probable Mechanism of Action of Diazene Antibiotics[1]

Edward M. Kosower* and Tetsuo Miyadera†

Department of Chemistry, State University of New York, Stony Brook, New York 11790. *Received August 6, 1971*

Compounds with the structure AN=NB (and the potential for conversion to that structure, as ANHNHB) are now recognized as a new class of biological control agents, the *diazene antibiotics*. Examples include the antibiotic, hexahydrospinamycin, the glutathione-oxidizing agents, "azoester" and "diamide." Within specific chemical classes of diazene antibiotics, higher antifungal activity can be correlated with higher rate of reaction with glutathione. Reasons are presented for the idea that the antibiotic action of diazenes may involve intracellular oxidation of glutathione to its disulfide, and compounds which could effect this conversion in a catalytic fashion (by cycling through reoxidizable hydrazo compounds) have been designed and synthesized.

Recognition of antibiotic classes serves to accelerate pursuit of effective agents and their mode of action. Three sources provided the stimulus for the classification of compounds with the structure AN=NB as *diazene antibiotics*. First, the antifungal activity of phenylthiosemicarbazide (**1**) derivatives has been known for many years (*cf.* references cited in reference 2). Recently, the diazenes derived by oxidation of **1** have been implicated as the active biological agents (*e.g.*, **2**).[3,‡] Second, reagents introduced by ourselves had clearcut antibiotic activities. These reagents included

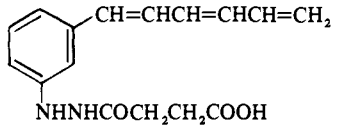
$C_6H_5NHNHCSNH_2$ (**1**) $C_6H_5N{=}NCSNH_2$ (**2**)

†On leave from Central Research Laboratories, Sankyo Co., Tokyo, Japan.

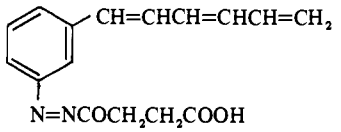
‡The care required to demonstrate the intermediacy of diazenes because of hydrolytic instability is not appreciated (*cf.* ref 3b).

$C_6H_5N{=}NCOOCH_3$ (3) $(CH_3)_2NCON{=}NCON(CH_3)_2$ (4)

methyl phenyldiazenecarboxylate ("azoester," **3**)[1,4] and diazenecarboxylic acid bis(*N,N*-dimethylamide) ("diamide," **4**).[4,5] Azoester inhibited the germination of the fungus, *Trichoderma viride*,[6] and diamide halted the growth of *Escherichia coli*.[7] Third, the discovery of the antibiotic spinamycin (**5**),[8,9] chemically analogous to the thiosemicarbaside **1** and capable of oxidation to the diazene **6**, prompted us to a consideration of possible common mechanisms of action for these 3 groups.

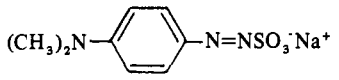


5

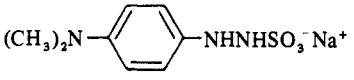


6

After we had completed most of the work described in this article, we came across a commercially used fungicide, Dexon (4-*N,N*-dimethylaminophenyldiazenesulfonic acid, sodium salt, **7**, available from Chemagro Corp., Kansas City, Mo.)[§,11] which, together with the corresponding hydrazine (**8**) should fit the general requirement for a molecule that might behave as a diazene antibiotic (see Discussion). The wide use of **7**[12] only underlines the necessity for a better understanding of the agents which we shall now refer to as *diazene antibiotics*.

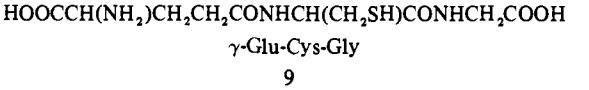


7



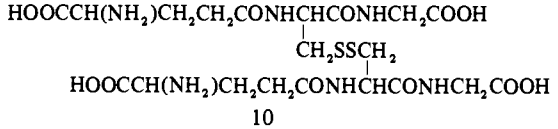
8

At this point, it should be clear that an empirical relationship based on structure exists among all the compounds we have mentioned. We now introduce the idea that the diazene agents, azoester and diamide (**3** and **4**), act inside the cell to oxidize the tripeptide thiol, glutathione (**9**) (GSH), to the disulfide (GSSG, **10**), according to the two-step mechanism shown in eq 1 and 2.[1,4]

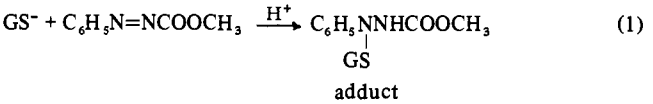


γ-Glu-Cys-Gly

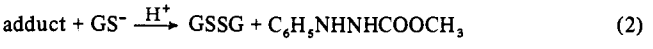
9



10

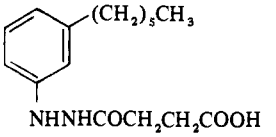


adduct



GSH is a "submajor" constituent of all cells; our recent studies have brought forward many indications that GSH is closely linked to many important intracellular processes.[1,4] It was thus natural to focus on the reaction between GSH and the diazenes as the logical basis of the antibiotic activity.

We therefore examined some derivatives of spinamycin. The triene antibiotic was somewhat too reactive toward air for our purposes and our studies have all been made with derivatives of hexahydrospinamycin (**11**), for which a convenient synthesis is described in the accompanying note.[13]

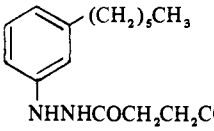


11

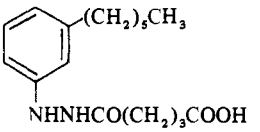
The objectives of the studies we have made included (a) obtaining evidence that diazenes rather than hydrazo compounds were invariably the active form of diazene antibiotics, (b) examining the effect of variation of the *N*-acyl group in hexahydrospinamycin and related compounds, (c) designing and synthesizing new members of the class of diazene antibiotics on the basis of principles we have deduced for them, and (d) introducing an objective rapid method for evaluating diazene antibiotics through measurement of the rate of reaction with GSH.

Results

Synthesis. In an effort to learn more about hexahydrospinamycin (**11**) behavior, a number of closely related compounds were prepared. The Me ester (**11-M**) was generated from CH_2N_2 and **11**, while the Et ester (**11-E**) was made from 3-*n*-hexylphenylhydrazine[13] and 2-carbethoxypropionyl chloride. The hydrazine was also treated with glutaric anhydride to form hexahydrohomospinamycin (**12**).

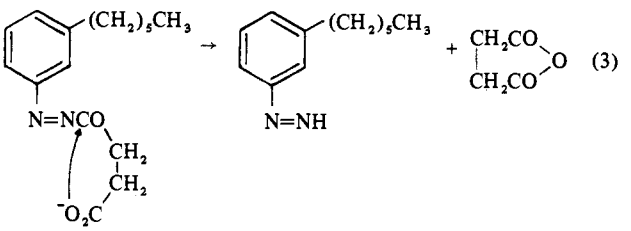


11-M



12

Attempts to oxidize **11** to the diazene derivative in CH_2Cl_2 led to an unstable product from which succinic anhydride and an oil, presumably *n*-hexylbenzene, could be isolated after treatment with H_2O. This result was consistent with intermediate formation of the diazene, but followed by intramolecular attack on the diazene carbonyl by the CO_2H group. Succinic anhydride and 3-*n*-hexylphenyldiazene should be the products of the intramolecular reaction[#] (eq 3). The diazene[15] is highly susceptible to oxidation by O_2 and thus yields the hydrocarbon in small amounts (eq 4).

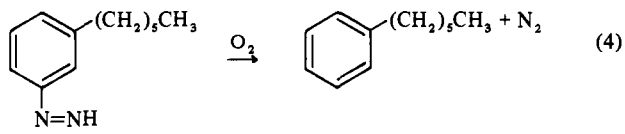


(3)

Phenyldiazene can be demonstrated as a product of water-induced decomposition of $C_6H_5N{=}NCOCH_2CH_2COOH$.

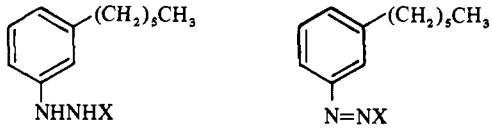
§Toxicity and pharmacological studies have been reported by Herrman and DuBois.[10]

#Diazenyl carbonyl groups (C=O next to N=N) are very readily attacked by nucleophiles (*cf.* ref 14).



(4)

Neighboring group participation by the carboxyl group precluded further work on the diazenes directly related to hexahydrospinamycin (**11**) even though it might have been possible to prepare water-soluble compounds with a less nucleophilic group in place of the CO_2H group (*e.g.*, SO_3H). A series of compounds analogous to **11** were prepared from 3-*n*-hexylphenylhydrazine by reaction with a carbamoyl chloride or with thiocyanate ion. These materials, the *N,N*-dimethylamide (**13**), the *N,N*-dimethylthioamide (**15**), and the thioamide (**17**), could be converted into the corresponding diazene derivatives (**14**, **16**) without special difficulty.



X

13, $CON(CH_3)_2$
15, $CSN(CH_3)_2$
17, $CSNH_2$

X

14, $CON(CH_3)_2$
16, $CSN(CH_3)_2$

Preliminary biological tests indicated that moderate quantities of hydrazine derivatives were required for antifungal action. The active species appeared to be the diazenes, in accordance with our previous work and the results reported by the Dutch group.[3] In studies on the mechanism of the reaction of GSH with diazene esters of the type, ArN=NCOOR, we had noted that the hydrazo forms were readily oxidized to the diazenes if the substituent on the ring were a strong electron-supplying group such as 4-Me_2N-.** We therefore conceived the idea of utilizing a system in which the biologically active diazene could be regenerated by reaction with O_2. The second product of the oxygen reaction may well be H_2O_2, which might be decomposed by catalase, by glutathione peroxidase, or in reactions with antibiotic consequences.

Based on these principles, we selected the 4-alkoxy group as a substituent which would (a) permit the GSH reaction with the diazene to proceed at a reasonable rate and (b) activate the ring sufficiently by electron supply so as to promote a reasonably fast return to the diazene with O_2. The cycle is illustrated in eq 5 and 6.

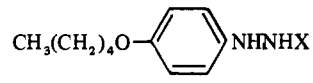
$$ArN{=}NX + 2GSH \rightarrow ArNHNHX + GSSG \quad (5)$$

$$ArNHNHX + O_2 \rightarrow ArN{=}NX + H_2O_2 \quad (6)$$

Preliminary biological tests in which we compared hexahydrospinamycin derivatives with ring-unsubstituted derivatives indicated that the long side chain played an important role in the antifungal action, possibly to promote entry into the fungal spore. Thus, in this reasonably logical way, we chose to prepare a series of derivatives of 4-pentyloxyphenylhydrazine, and were able to obtain both the hydrazo compounds (**18**, **20**, **22**) and the diazene derivatives (**19**, **21**, **23**). For comparison purposes, we also made two unsubstituted analogs (**24** and **25**) in addition to **2**.

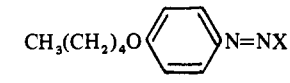
The synthetic procedures were, in general, straightforward, although much care (and sometimes, partial exclusion of air) is required in working with arylhydrazines and their derivatives, especially those with electron-supplying substituents on the ring.

**E. M. Kosower and W. Correa, unpublished results.



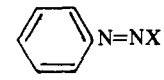
X

18, $CON(CH_3)_2$
20, $CSN(CH_3)_2$
22, $CSNH_2$



X

19, $CON(CH_3)_2$
21, $CSN(CH_3)_2$
23, $CSNH_2$



X

2, $CSNH_2$
24, $CON(CH_3)_2$
25, $CSN(CH_3)_2$

Biological Tests. For screening purposes, a very simple fungal growth test was utilized. Incubation of a weighed soil sample in minimal medium was carried out in the presence of various concentrations of antifungal agent. Primary information came from tests with $10^{-4}M$ agent, as summarized in Table I. Comparisons among the most active compounds were made for $10^{-5}M$ agent (Table II), with certain compounds showing activity even at $10^{-6}M$. Although these tests only apply to a particular local "natural" selection of fungal spores, the results are clear enough to justify further investigations.

The most active agents were the *N,N*-dimethylthioamides (**16**, **20**, **21**) bearing a hydrophobic group on the benzene ring.

Kinetic Results. To obtain an objective measure of the reactivity of the various derivatives we have described, we have examined the rates of reactions of the diazene derivatives with GSH in neutral aq soln. All reactions were carried out in the absence of O_2. The rate constants for the systems examined are listed in Table III.

Spectroscopic Results. Several points are worth noting about the spectra of the *N,N*-dimethylamides and *N,N*-dimethylthioamides. The uv spectra of 4-pentyloxyphenyldiazine derivatives are almost independent of the substituent on the diazene group (**19**, **21**, **23**). In MeCN, λ_{max} for **21** are 247.5 (ϵ_{max} 18,700) and 324.0 (16,000) (in nm). The $n \rightarrow \pi^*$ transition appears only as a shoulder between 400 and 450 nm.

The *N,N*-dimethylamide and thioamide groups exhibit 2 different Me shifts in their nmr, with the separation between the positions of the Me groups in the thioamide case much greater than that in the amide. Values for **19** and **21** illustrate the point. Such differences in local shielding have been noted previously.[16] (**19**) $(CH_3)_2NCO$, τ 6.97 and 7.07, CH_3 (of C_5H_{11}), τ 9.07; (**21**) $(CH_3)_2NCS$, τ 6.57 and 6.91, CH_3 (of C_5H_{11}), τ 9.06.

Discussion

The biological activity of a particular substance depends upon a complex sum of individual properties including affinity for the target site, survival in the medium of application, survival within the biological system, transport properties, and state of the target organism. It follows that many comparisons are only valid between very closely related, *i.e.*, *cognate* compounds. The conclusions we will draw from our results will be based on pairs of cognate compounds.

In comparisons of hydrazo (NHNH) and diazene (N=N) compounds for antifungal activity (Table I) it is apparent that the oxidized form is always as active or more active than the reduced form. In addition, a yellow color suggesting some formation of diazene appeared in the antifungal

Table I. Antifungal Activity of Diazene Derivatives[a,b]

Basic structure	Compound derivative designation	Extent of growth, scale (0–6+)[c] Time, hr 21	39	65	87	115[d]
m-$CH_3(CH_2)_5C_6H_4NHNHCOCH_2CH_2$	COOH (11)	0	+1	+2	>+2	>+2
	$COOCH_3$ (11M)	0	+1	+2	+2	+2
	$COOCH_2CH_3$ (11E)	0	0	+2	+2	+2
	CH_2COOH (12)	0	+1	+2	+2	+2
m-$CH_3(CH_2)_5C_6H_4NHNH$	$CON(CH_3)_2$[e] (13)	+1	+2	+4	+6	+6
	$CSN(CH_3)_2$[e] (15)	0	+1	+2	+2	+3
	$CSNH_2$[e] (17)	0	+1	+2	+2	+3
m-$CH_3(CH_2)_5C_6H_4N{=}N$	$CON(CH_3)_2$ (14)	+1	+2	+2	+2	+2
	$CSN(CH_3)_2$[e] (16)	0	+1	+1	+2	+2
p-$CH_3(CH_2)_4OC_6H_4NHNH$	$CON(CH_3)_2$[e] (18)	0	+2	+3	+3	+3
	$CSN(CH_3)_2$ (20)	0	0	+1	+2	+2
	$CSNH_2$ (22)	0	+2	+3	+4	+4
p-$CH_3(CH_2)_4OC_6H_4N{=}N$	$CON(CH_3)_2$ (19)	0	+1	+2	+3	+3
	$CSN(CH_3)_2$ (21)	0	+1	+2	+2	+2[f]
	$CSNH_2$ (23)	0	+2	+2	+2	+2
$C_6H_5N{=}N$	$CON(CH_3)_2$ (24)	+1	+2	+3	+4	+4
	$CSN(CH_3)_2$ (25)	0	+2	+2	+2	+3
	$CSNH_2$ (2)	+1	+2	+4	+6	+6
Control		+2	+3	+4	+6	+6

[a]To a 0.1% soln of KH_2PO_4, NH_4Cl, and glucose in distd H_2O, soil was added (1 g/30 ml), the mixt was swirled 20 times, and the agent to be tested was added, either as a solid or as a concd soln in EtOH, to give a gross concn of 10^{-4} M. Results similar to those in the Table were obtd for a medium contg 1% glucose. [b]Dihydrodiazenes = diazanes = hydrazo compounds. [c]The scale used was qualitative. On the basis of fungal growth observed, results were scored as follows, with the control as a guide: 0–no growth; +1–very little growth; +2–little growth; +3–growth; +4–reasonable growth; +5–substantial growth; +6–much growth (= control at maximum). [d]Little change was observed after this time. [e]Not all material was dissolved. [f]Most active compound of all tested.

Table II. Comparison of Effective Antifungal Agents[a]

Substance[b]	Extent of growth,[c] scale (0–6+) Time, hr 21	39	65	87
11	+2	+2	+4	+6
11-M	+1	+2	+3	+6
11-E	+1	+2	+4	+6
12[f]	0	+2	+4	+6
14	+2	+2	+3	+6
16[f]	0	+2	+2	+2
20[d,f]	0	+1	+1	+2
21[e,f]	0	+2	+2	+2
23	0	+1	+2	+4
Control	+2	+3	+4	+6

[a]Tests were carried out as described in footnote *a*, Table I, except that the concs of agents were 10^{-5} M. [b]For formulas, see Table I. [c]The scales are described in footnote *c*, Table I. [d]Appeared to be the second most active substance tested. [e]Most active substance tested. [f]Exhibited definite activity at 10^{-6} M.

Table III. Rate Constants for Reaction of Diazene Derivatives with Glutathione (GSH) in 30% Ethanol[a,b]

YArN=NX Y =	X =	pH[c]	λ,[d] nm	k,[e] M^{-1} sec^{-1}
H	$CSNH_2$ (2)	7.65	400	2.05
H	$CSN(CH_3)_2$ (25)	7.50	350[f]	3.77×10^{-2}
H	$CON(CH_3)_2$ (24)	[g]	430[f]	1.25×10^{-3}[h]
n-C_5H_{11}	$CSNH_2$ (23)	7.65	355[f]	0.252
n-$C_5H_{11}O$	$CSN(CH_3)_2$ (21)	7.65	350	1.28×10^{-3}
n-C_6H_{13}	$CSN(CH_3)_2$ (16)	7.60	375	7.15×10^{-3}
$(CH_3)_2N$	$SO_3^-Na^+$ (7)	7.24[i]	495	$<10^{-5}$[j]

[a]Reaction mixts were degassed by alternate partial evacuation and filling with N_2. Temp 25 ± 1°. [b]Phosphate buffer (0.5 M, pH 7.24) was added to 3.0 ml of an EtOH soln of the diazene derivative until the total vol was 10.0 ml. After deoxygenation, the soln was poured onto a weighed amount of GSH contd within the apparatus. Recording of optical density could usually be started within 15–30 sec. [c]Measured at the end of the kinetic run for the reaction mixt itself. [d]Wavelength at which the reaction mixt was followed. [e]Calcd with a program for the bimolecular reaction, A + 2B. Rate constants are ±5%. In all cases but the one noted in footnote *h*, the GSH concn exceeded that of the diazene by at least a factor of 10. [f]Absorption maximum (in nm). [g]Between 7.50 and 7.60. [h][GSH] 1.83×10^{-2} M; [Diazene] 3.79×10^{-3} M (the GSH concn was limited by the buffer concn used). [i]Aq buffer was used as solvent. [j]Not more than 1% reaction was observed spectroscopically in a soln contg 0.01 M GSH.

test solution of hydrazo compounds. It seems likely that the diazene derivative is the active form of these antibiotic substances.

In Table IV, we have assembled our limited data on cognate pairs of diazene compounds. Cognates containing either $CSN(CH_3)_2$ or $CON(CH_3)_2$ are compared in rate of reaction with GSH and antifungal activity. The thioamides are always higher in antifungal activity and more reactive toward GSH, as shown by the pairs **25/24**, **21/19**, and **16/14**.

Another cognate pair (**2/23**) cited in Table IV is one in which an H on the aromatic ring is replaced by a 4-pentyloxy group. In this case, it appears that hydrolysis of the agent over the long periods during which an antifungal test is conducted causes its antifungal activity to be lower than one might have expected. It is also possible that the lack of a hydrophobic group on the ring lowers the rate of entry of the agent into the fungal spore.

From these facts, we derive the tentative generalization that reaction of diazene derivatives (including hexahydrospinamycin and spinamycin) with GSH within the cell is responsible for the antibiotic activity.††

We may now consider how the intracellular oxidation of GSH to GSSG might affect the growth of a fungal spore or other cells. The action of the diazene antibiotic, diamide (**4**), on rabbit reticulocytes causes (a) oxidation of GSH and (b) immediate cessation of protein synthesis.[1] After the intracellular GSH has been substantially regenerated, translation of mRNA is completed but only after practically all of the original GSH is present does initiation of protein synthesis recover to its usual level. The sensitivity of a portion of the protein synthesizing apparatus to mild oxi-

††Trichloromethyl sulfenyl fungicides (*e.g.*, captan, folpet) may also form GSSG from GSH in reactions analogous to those for diazenes.[17]

Table IV. Comparison of Diazene Reactivities and Antifungal Activities

No.	(YArN=NX) Y	X	$10^3 k_2$,[a] M^{-1} sec^{-1}	Antifungal activity score[b]
2	H	$CSNH_2$	2050	16
23	$C_5H_{11}O$	$CSNH_2$	252	8
25	H	$CSN(CH_3)_2$	38	9
24	H	$CON(CH_3)_2$	1.3	14
21	$C_5H_{11}O$	$CSN(CH_3)_2$	1.3	7
19	$C_5H_{11}O$	$CON(CH_3)_2$	(0.04)[c]	11
16	C_6H_{13}	$CSN(CH_3)_2$	7.2	6
14	C_6H_{13}	$CON(CH_3)_2$	(0.24)[c]	9

[a]Rate constants for reaction with GSH in 30% EtOH. [b]Sum of numbers for growth scores recorded in Table I; the higher the number, the lower the antifungal activity. [c]Estimated from rate constant ratio 25/24.

dation is illustrated by the remarkable effect of GSSG itself on protein synthesis in a lysate. GSSG in the presence of a large excess of GSH turns off protein synthesis, with the initiation implicated as the sensitive site.[18,19] The importance of GSH to the germination of spores has been shown by Richmond and Somers for *Neurospora crassa*[20] for which GSH synthesis precedes protein synthesis.

Oxidation of GSH within cells (spores) provides a mechanism for arresting growth. In the absence of additional challenge this arrestation of growth is reversed when the GSH:GSSG ratio has returned to an appropriate ratio. If, however, an additional challenge is introduced, as is the case for azoester 3 which yields free radicals (eq 7 and 8)

$$C_6H_5N{=}NCOOCH_3 + H_2O \rightarrow C_6H_5N{=}NCOOH \rightarrow C_6H_5N{=}NH \quad (7)$$

$$C_6H_5N{=}NH + O_2 \rightarrow C_2H_5\cdot + N_2 + ?\cdot OOH \quad (8)$$

in addition to oxidizing GSH to GSSG, a bactericidal or fungicidal effect is seen.[1,4,6] The free radicals, in most cells, cause peroxidation processes to be initiated in the membrane, thus rendering the membrane more susceptible to lysis.[1,‡‡]

The low rate of reaction of Dexon (7) with GSH indicates quite clearly that the antifungal activity does not arise from the same source as that of the other diazene antibiotics. The technical information bulletin (available from Chemagro Corp., Kansas City, Mo.) suggests that phosphorylation is inhibited. A possible course for this inhibition involves formation of a phosphorylated 7 (eq 9), ionization of the phosphosulfite, and reaction of the diazonium ion with a tyrosine or tryptophan of the protein of the phosphorylating protein (eq 10, 11).

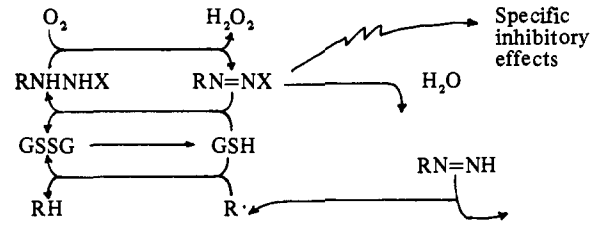
$$(CH_3)_2NC_6H_4N{=}NSO_3^- \rightarrow (CH_3)_2NC_6H_4SO_3PO_3^{--} \quad (9)$$

$$(CH_3)_2NC_6H_4N{=}NSO_3PO_3^{--} \rightarrow (CH_3)_2NC_6H_4N{\equiv}N^+ + (SO_3PO_3)^{-3} \quad (10)$$

$$(CH_3)_2NC_6H_4N{\equiv}N^+ + HOC_6H_4\text{-protein} \rightarrow \text{azoprotein} \quad (11)$$

The scheme (I) below summarizes our present view of the activities of diazene antibiotics. The cyclic reactions shown can operate independently of many other types of antibiotic activities and the scheme suggests that diazene antibiotics might be synergists for other antibiotics.

Scheme I



Experimental Section

Nmr spectra were taken with a Varian A-60 spectrometer. Uv and visible spectra (and all kinetic measurements) were determined with a Cary Model 14 spectrophotometer. Structures were confirmed by nmr.

Kinetic Measurements. Rate measurements were carried out with solns prep as described in footnotes *a* and *b*, Table III, in a quartz cell attached by a graded seal to a Pyrex tube bearing a 3-way stopcock and a small side-arm into which solid samples (or even solutions) could be introduced and maintained during the deoxygenation procedure. Reactions were followed at the wavelength cited and then by taking complete spectra (if the rates were low enough). Optical densities were taken for different times and the rate constants calculated for a second-order reaction in which a second molecule of GSH is consumed after the rate-limiting step. An IBM 1800 computer was used for the calculation of most of the constants, and a GE 635 computer was used for the remainder; 30% EtOH was chosen as a solvent with sufficient water content to resemble aqueous solutions but with enough organic content to dissolve the diazene derivative used. A number of rates were examined in solvents containing much more H_2O (*i.e.*, 6% MeCN) but the rate constants only differed by the factor expected for the measured difference in pH (pH rises as org sol is added to phosphate buffer).

Biological Tests. A 0.1% soln of each of KH_2PO_4, NH_4Cl, and dextrose in distd H_2O was prepd. Soil (1 g) was added to 30 ml of each soln, and the mixt was swirled 20 times. The solns contd amounts of the substances to be tested so as to have a gross final concn of 10^{-4}, 10^{-5}, and 10^{-6} *M*. Complete soln was not attained in many cases, especially for the higher concns. A number of the hydrazo compds (colorless or faintly colored compds) yielded suspensions which had distinct yellow colors, suggesting that oxidation to the corresponding diazene had occurred. The tests were scored qualitatively as indicated in footnote *c*, Table I, with emphasis placed on extent and area of mycelial growth. Further tests against specific fungi would be desirable, but a survey of this type is not presently possible for us.

2-*N*-Glutaroyl-1-*N*-(3-*n*-hexylphenyl)hydrazine (12). 3-*n*-Hexylphenylhydrazine (384 mg) and glutaric anhydride (228 mg) in PhH[21] (10 ml) were stirred overnight, the solvent was evapd, and the residue was washed (H_2O) and recrystd from $CHCl_3$–CCl_4 to yield colorless, fine crystals (215 mg, 36%), mp 87.5–88.5°.

Me Ester of Hexahydrospinamycin (11M). Hexahydrospinamycin (490 mg) in Et_2O was treated with ethereal CH_2N_2 at room temp, the solvent evapd, and the residue recrystd from CCl_4–hexane to give colorless, fine needles (410 mg), mp 69–70°.

Et Ester of Hexahydrospinamycin (11E). 2-Carbethoxypropanoyl chloride (219 mg) in anhyd Et_2O (10 ml) was added to an ice-cooled soln of 3-*n*-hexylphenylhydrazine (511 mg) in Et_2O (10 ml). Pyridine (210 mg) in Et_2O (10 ml) was added, followed by the remainder of the acid chloride (219 mg) in Et_2O (10 ml). After stirring for 10 min, H_2O and dil HCl were added, the Et_2O layer was sepd, washed with H_2O, aq $NaHCO_3$ and then H_2O, and evaporated, and the residue chromatogd on silica gel (60–200 mesh) in hexane. The Et ester was eluted with PhH–EtOAc (5:1) to give an oil (490 mg) which was crystd from CCl_4–hexane, affording colorless fine needles, mp 70–71°.

4-*n*-Pentyloxyphenylhydrazine Hydrochloride. 4-Pentyloxyphenylaniline (3.62 g) in concd HCl (5.0 ml) and H_2O (5.0 ml) was diazotized at 0–5° with a soln of $NaNO_2$ (1.38 g) in H_2O (2 ml). The soln of diazonium salt was added to a soln of $SnCl_2$ (11.4 g) in concd HCl (50 ml) at 0–5°. Et_2O (100 ml) was added and the mixt stirred for 30 min. The ppt of product was filtered off and washed (H_2O, Et_2O) to give almost pure crystals (3.11 g), mp 168°. Recrystn from EtOH gave colorless crystals, mp 169–170°.

2-*N*-(4-*n*-Pentyloxyphenyl)-1-*N*-(thiocarbamoyl)hydrazine (22). A suspension of 4-pentyloxyphenylhydrazine hydrochloride and KSCN (0.583 g) in EtOH (10 ml) was refluxed with stirring for 24 hr.[22] The reaction mixt was poured into ice-water and the ppt filtered off. Recrystn from PhH gave colorless crystals (352 mg), mp 164–165°.

1-*N*-(4-*n*-Pentyloxyphenyl)-2-*N*-(*N*,*N*-dimethylthiocarbamoyl)-hydrazine (20). 4-*n*-Pentyloxyphenylhydrazine (1.858 g) (from the hydrochloride and aq NaOH) was dissolved in anhyd Et_2O (20 ml) and dimethylthiocarbamoyl chloride (0.604 g) added. The mixt

‡‡E. M. Kosower, N. S. Kosower, and E. L. Gottfried, unpublished results.

was stirred for 48 hr at room temp. The pptd hydrazine hydrochloride was filtered off, the solvent evapd, and the residue chromatogd on silica gel in PhH, after elution with PhH. The product was eluted with PhH–EtOAc (9:1). The oily product was crystd from hexane and recrystd from EtOAc–hexane to give colorless crystals (140 mg), mp 103–104°.

1-*N*-(4-*n*-Pentyloxyphenyl)-2-(*N*,*N*-dimethylcarbamoyl)hydrazine (18). A mixt of 4-*n*-pentyloxyphenylhydrazine hydrochloride (1.153 g) and dimethylcarbamoyl chloride (0.538 g) in pyridine (20 ml) was allowed to stand at room temp for 24 hr, the solvent evapd *in vacuo*, and H_2O added. The ppt was filtered off, washed (H_2O), and dried. Recrystn from Et_2O gave colorless crystals (542 mg), mp 135–136°.

4-*n*-Pentyloxyphenyldiazenecarboxylic Acid Thioamide (23). *p*-Benzoquinone (54 mg) in MeOH (5 ml) was added at room temp to a soln of **12** (127 mg) in MeOH (20 ml). The mixt was stirred for 30 min and poured into ice water, and the ppt (125 mg) was collected, mp 100–101°. Recrystn from PhMe at −78° gave red crystals, mp 100–101°. The *N*,*N*-dimethylthioamide (**21**), orange crystals from hexane, mp 83–84° (80% yield), was obtained in a similar manner.

4-*n*-Pentyloxyphenyldiazenecarboxylic Acid *N*,*N*-Dimethylamide (18). *N*-Bromosuccinimide (239 mg) in CH_2Cl_2 (20 ml) was added with cooling in Dry Ice–acetone bath to a soln of **14** (356 mg) and pyridine (110 mg) in CH_2Cl_2 (10 ml). The mixt was stirred for 30 min and for an addl hr after Dry Ice–acetone bath was removed. The reaction mixt was washed (H_2O) and dried (Na_2SO_4), and the solvent was evapd. The residue was recrystd from Et_2O (below −70°) to give orange crystals (263 mg), mp 56–57°.

1-*N*-(3-*n*-Hexylphenyl)-2-(*N*,*N*-dimethylcarbamoyl)hydrazine (13). A soln of *m*-*n*-hexylphenylhydrazine (762 mg) and dimethylcarbamoyl chloride (213 mg) in Et_2O (10 ml) was stirred for 20 hr at room temp. The solvent was evapd, PhH added, and the insol *m*-*n*-hexylphenylhydrazine hydrochloride filtered off. The filtrate was concd and the product recrystd from the soln to give colorless crystals (160 mg), mp 156–157°. The corresponding diazene (**10**) was generated from **9** by a procedure like that used for **18**. Chromatography on silica gel in hexane and elution with PhH gave a reddish oil, which was shown to be a pure sample of the expected product (**13**) by tlc and nmr.

Acknowledgments. The authors are grateful to the Army Research Office (Durham, N. C.) and the National Institutes of Health for support.

References

(1) T. Zehavi-Wilner, E. M. Kosower, T. Hunt, and N. S. Kosower, *Biochim. Biophys. Acta*, 228, 245 (1971) (part 5); N. S. Kosower, *et al.*, *ibid.*, 192, 1, 8, 15, 23 (1969).
(2) H. G. Mautner and W. D. Kumler, *J. Amer. Chem. Soc.*, 78, 97 (1956).
(3) (a) C. W. Pluijgers, J. Berg, A. Kaars Sijpesteijn, A. Tempel, and A. Verloop, *Recl. Trav. Chim. Pays-Bas*, 87, 833 (1968); (b) A. Fuchs, A. L. Homans, and F. W. DeVries, *Ann. Appl. Biol.*, 65, 489 (1970).
(4) E. M. Kosower and N. S. Kosower, *Nature (London)*, 224, 117 (1969).
(5) N. S. Kosower, E. M. Kosower, B. Wertheim, and W. Correa, *Biochem. Biophys. Res. Commun.*, 37, 593 (1969).
(6) D. Weinman, E. Galun, N. S. Kosower, and E. M. Kosower, *Experientia*, 26, 40 (1970).
(7) R. Wax, E. Rosenberg, N. S. Kosower, and E. M. Kosower, *J. Bacteriol.*, 101, 1092 (1970).
(8) E. L. Wang, M. Hamada, Y. Okami, and H. Umezawa, *J. Antibiot., Ser. A.*, 19, 216 (1966).
(9) H. Naganawa, T. Takita, K. Maeda, and H. Umezawa, *ibid.*, 21, 241 (1968).
(10) R. G. Herrman and K. P. DuBois, *J. Pharmacol. Exp. Ther.*, 95, 262, 272 (1949).
(11) E. Urbschat and P. E. Frohberger, U. S. Patent 2,911,336; *Chem. Abstr.*, 54, 20056 (1960).
(12) G. C. Papavizas, *Plant Dis. Rep.*, 51, 125 (1967); W. Krueger, *Phytopathol. Z.*, 62, 174 (1968); J. T. Mills and H. A. Wallace, *Can. J. Plant Sci.*, 48, 587 (1968); H. Cole, D. Mackenzie, and C. B. Smith, *Bull. Environ. Contam. Toxicol.*, 3, 116 (1968); N. H. Peck, B. E. Clark, and J. J. Natti, *Agron. J.*, 59, 78 (1967).
(13) T. Miyadera and E. M. Kosower, *J. Med. Chem.*, 15, 339 (1972).
(14) P.-K. C. Huang and E. M. Kosower, *J. Amer. Chem. Soc.*, 90, 2354 (1968).
(15) E. M. Kosower, *Accounts Chem. Res.*, 4, 193 (1971).
(16) R. C. Neuman, Jr., and L. B. Young, *J. Phys. Chem.*, 69, 1777 (1965).
(17) M. R. Siegel, *J. Agr. Food Chem.*, 18, 819 (1970); E. Somers, Abstracts, 156th Meeting of the American Chemical Society, Atlantic City, N. J., AGFD 72 (1968).
(18) N. S. Kosower and E. M. Kosower, *Lancet*, ii, 1343 (1970).
(19) N. S. Kosower, G. Vanderhoff, B. Benerofe, T. Hunt, and E. M. Kosower, *Biochem. Biophys. Res. Commun.*, 45, 816 (1971).
(20) D. V. Richmond and E. Somers, *Biochem. Biophys. Res. Commun.*, 25, 657 (1966).
(21) Cf., J. Buechi, H. Muehle, H. Braunschweiger, and P. Fabiani, *Helv. Chim. Acta*, 45, 449 (1962).
(22) Cf., Th. Pyl, K. H. Scheel, and H. Beyer, *J. Prakt. Chem.*, 20, 255 (1963).

Organic Disulfides and Related Substances. 33. Sodium 4-(2-Acetamidoethyldithio)butanesulfinate and Related Compounds as Antiradiation Drugs[1,†]

Lamar Field* and Yong H. Khim

Department of Chemistry, Vanderbilt University, Nashville, Tennessee 37203. *Received September 6, 1971*

The compound $AcNH(CH_2)_2SS(CH_2)_4SO_2Na$ (**1**), a promising antiradiation drug, disproportionates far more rapidly in solution to the symmetrical disulfides **2** (the amide) and **3** (the sulfinate) than would be expected from behavior of analogous compounds having no SO_2Na moiety; solid **1** is stable. The disproportionation is reversible. An improved preparation of **1** minimizes disproportionation by use of lower temperatures, precipitation instead of concentration, and minimum contact with polar solvents. Radioprotective activity of improved **1** confirms earlier results; **3** is active as well, but **2** is inactive. The trisulfide $[NaO_2S(CH_2)_4S]_2S$ (**9**) was prepared in 80% yield from 1,2-dithiane 1,1-dioxide (**6**) using Na_2S. Thus far, **9** has shown $ALD_{50} > 900$ mg/kg (ip or po) and 73–100% protection of mice at doses of 38 (ip)–300 (po) mg/kg in mice. The promising activity of **3** and (especially) **9** is noteworthy since neither contains a nitrogen function.

Sodium 4-(2-acetamidoethyldithio)butanesulfinate (**1**) has given promising results as an antiradiation drug.[2] However, difficulties were encountered in the large-scale preparation of pure **1** and in reproducing the promising protective activity using **1** that was obtained.‡ Disproportionation of **1** seemed

†This investigation was supported by the U. S. Army Medical Research and Development Command, Department of the Army, under Research Contract No. DADA17-69-C-9128.

‡Discussion with Dr. E. A. Steck of the Walter Reed Army Institute of Research, Washington, D. C.