Studies concerning the Antibiotic Actinonin. Part VIII.[1] Structure–Activity Relationships in the Actinonin Series

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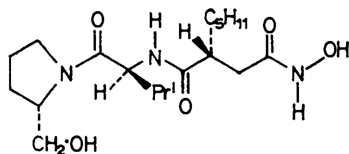
The *in vitro* activity of a series of actinonin derivatives against *S. aureus* has been correlated with free-energy-related substituent parameters by multiple regression analysis. The activity shows a parabolic dependence on partition which probably reflects transport and membrane permeability factors. The relationships also show a dependence on the steric properties of the side-chains and it is suggested that biological activity may be associated with the formation of a tetrahedral transition state at the hydroxamic acid carbonyl group. *In vivo* activity is poor, possibly because of metabolism to inactive cleavage products.

THE antibiotic actinonin (I) has been isolated from strains of *Actinomyces*[2] and *Streptomyces*.[3] The constitution has been determined by spectroscopic and degradative studies[3] and confirmed by total synthesis.[4]



(I)

Actinonin is active *in vitro* against Gram-positive and Gram-negative bacteria and also a number of phage strains,[2] but the antibacterial activity *in vivo* is poor and analogues were prepared in the hope of improving this property. Preliminary studies indicated that modification of the hydroxamic acid function or replacement by amide, hydrazide, carboxylic acid, nitrile, or imino-ether results in total loss of antibacterial activity. However, provided that the pseudopeptide backbone is retained, other modifications may be made without losing activity. The analogues in which the L-prolinol residue is replaced by a symmetrical substituent possess two centres of chirality and accordingly form two diastereoisomeric series. The series which is topographically equivalent to natural actinonin has the greater antibacterial activity, and the relationships between activity and structure of this series form the subject of this paper.

EXPERIMENTAL

Most of the required compounds were prepared with a high degree of stereochemical specificity by the anhydride–imide route and are described in earlier parts of this series.[5-7] The synthesis of the remaining compounds is described below.

3-(3-*Methyl-2-morpholinocarbonylbutyramido*)*octanohydroxamic acid* (21) (*Table* 2).—Morpholine (14·5 g) was added during 20 min to ethyl 2-chloroformyl-3-methylbutanoate[8] (16 g) in anhydrous chloroform (100 ml) at 0—5° and the mixture was kept at room temperature overnight.

[1] Part VII, N. H. Anderson, J. P. Devlin, S. Jones, W. D. Ollis, and J. E. Thorpe, preceding paper.
[2] J. J. Gordon, B. K. Kelly, and G. A. Miller, *Nature*, 1962, **195**, 701.
[3] Part I, J. J. Gordon, J. P. Devlin, A. J. East, W. D. Ollis, I. O. Sutherland, D. E. Wright, and L. Ninet, *J.C.S. Perkin I*, 1975, 819.
[4] Part II, N. H. Anderson, W. D. Ollis, J. E. Thorpe, and A. D. Ward, *J.C.S. Perkin I*, 1975, 825.
[5] Part III, J. P. Devlin, W. D. Ollis, J. E. Thorpe, R. J. Wood, B. J. Broughton, P. J. Warren, K. R. H. Wooldridge, and D. E. Wright, *J.C.S. Perkin I*, 1975, 830.
[6] Part IV, B. J. Broughton, P. J. Warren, K. R. H. Wooldridge, D. E. Wright, W. D. Ollis, and R. J. Wood, *J.C.S. Perkin I*, 1975, 842.
[7] Part VI, J. P. Devlin, W. D. Ollis, J. R. Thorpe, and D. E. Wright, *J.C.S. Perkin I*, 1975, 848.
[8] G. B. Brown and M. F. Ferger, *J. Amer. Chem. Soc.*, 1946, **68**, 1507.

The solution was washed successively with 0·2N-hydrochloric acid, saturated aqueous sodium hydrogen carbonate, and water, and then concentrated to give *ethyl* 3-*methyl*-2-*morpholinocarbonylbutyrate* (16·5 g, 82%) (Found: C, 59·1; H, 8·8; N, 5·5. $C_{12}H_{21}NO_4$ requires C, 59·3; H, 8·6; N, 5·8%). The ester (14 g) was warmed with 2N-sodium hydroxide (60 ml) until a clear solution was obtained. The pH was brought to 2 with 2N-hydrochloric acid and the mixture was continuously extracted with ether. Concentration of the extract afforded 3-*methyl*-2-*morpholinocarbonylbutyric acid* (7·7 g, 62%), m.p. 132—134° (Found: C, 56·0; H, 8·0; N, 6·4. $C_{10}H_{17}NO_4$ requires C, 55·8; H, 7·9; N, 6·5%).

The acid (2·15 g), ethyl 3-amino-octanoate[9] (1·87 g), and dicyclohexylcarbodi-imide (2·06 g) in anhydrous methylene chloride (50 ml) were refluxed for 3 h. Filtration and concentration afforded *ethyl* 3-(3-*methyl*-2-*morpholinocarbonylbutyramido*)*octanoate* (3·2 g, 84%), m.p. 94—95° [from petroleum (b.p. 60—80°)] (Found: C, 62·4; H, 9·6; N, 7·2. $C_{20}H_{36}N_2O_5$ requires C, 62·5; H, 9·4; N, 7·3%).

The ester (3·84 g) in methanol (40 ml) was added to a solution of hydroxylamine in methanol [from hydroxylamine hydrochloride (1·39 g), potassium hydroxide (1·68 g), and methanol (20 ml)] and kept at room temperature overnight. The mixture was then evaporated to give an oil which was dissolved in water; the solution was extracted with chloroform. Chromatography of the extract (silica gel; chloroform–acetone) afforded *compound* (21) (1·0 g), m.p. 170—173° (Found: C, 58·5; H, 8·6; N, 11·2. $C_{18}H_{33}N_3O_5$ requires C, 58·2; H, 8·9; N, 11·3%).

6-*Methyl*-3-(3-*methyl*-2-*morpholinocarbonylbutyramido*)-*heptanamide* (22) (*Table* 2).—DL-2-(3-Methylbutyl)-*N*-(2-methyl-1-morpholinocarbonylpropyl)succinimide[5] (1·5 g) in methanol (30 ml) saturated with anhydrous ammonia was heated in a Carius tube at 100° for 20 h. The mixture was then evaporated to dryness and the residue dissolved in chloroform. The solution was washed successively with saturated aqueous sodium hydrogen carbonate, 2N-hydrochloric acid, and water. Concentration afforded the *amide* (22) (0·55 g, 33%), m.p. 173—175° (from ethyl acetate) (Found: C, 61·0; H, 9·6; N, 11·7. $C_{18}H_{33}N_3O_4$ requires C, 60·8; H, 9·4; N, 11·8%).

6-*Methyl*-3-(3-*methyl*-2-*morpholinocarbonylbutyramido*)-*heptanohydrazide* (23) (*Table* 2).—A solution of hydrazine in methanol [from hydrazine hydrochloride (0·2 g), potassium hydroxide (0·25 g), and methanol (20 ml)] was added to DL-2-(3-methylbutyl)-*N*-(2-methyl-1-morpholinocarbonylpropyl)succinimide (0·5 g). The mixture was kept overnight at room temperature, then concentrated to dryness to give an oil which on trituration with petroleum (b.p. 40—60°) and ethyl acetate afforded the *hydrazide* (23) (0·090 g, 17%), m.p. 128—130° (from ethyl acetate) (Found: C, 58·6; H, 9·3; N, 14·9. $C_{18}H_{34}N_4O_4$ requires C, 58·4; H, 9·3; N, 15·1%).

Microbiological Methods.—In vitro *determination of antibacterial activity*. Solutions (0·1%) of compounds were prepared in neat propylene glycol; the minimum of heat was used to effect dissolution. Actinonin or Penicillin G were used as reference compounds. Serial two-fold dilutions were made in sulphonamide-testing broth usually ranging from 100 to 0·19 μg ml^{-1}. The broth dilutions were inoculated with log phase cultures of *Staphylococcus aureus* (NCTC 6571A) (Oxford strain) and *Escherichia coli* (NCTC 4144); *ca.* 100 cells were used per ml of broth. After overnight incubation at 37 °C the results were read as the Minimal Inhibitory Concentration (MIC) (μg ml^{-1}) according to the visible growth in comparison with broth without drug.

In vivo/in vitro *determination of antibacterial activity*. Groups of five albino mice (weight 20 g) were dosed orally with tragacanth mucilage suspensions of the trial compounds at 250 mg per kg body weight. Heart blood was removed under chloroform anaesthesia 1 h after treatment. The mixed blood from each group was tested *in vitro* by using two-fold serial dilutions prepared in 'Repli-dishes' with S.T. agar and running a duplicate series for each group. One series was infected with staphylococci and the other with the *Escherichia*, both from log phase broth cultures. After overnight incubation the antibacterial activity was read as the Maximum Inhibitory Dilution (MID).

In vivo *determination of antibacterial activity*. Albino mice (15—19 g) intraperitoneally infected with *ca.* 100 Minimal Lethal Doses (MLC) of *Staphylococcus aureus* (Smith) or *Escherichia coli* (NCTC 4144) were treated subcutaneously with suspensions of the compounds in tragacanth mucilage. The mice with staphylococcal infection were given a dose of 500 mg per kg body weight 0, 1, 3, and 24 h after infecting. The mice infected with *E. coli* survived less than 24 h and were dosed 0, 1, and 3 h after infecting.

RESULTS AND DISCUSSION

The results of the *in vitro* and *in vivo/in vitro* tests against *S. aureus* and *E. coli* (Tables 1—3) were considered in the light of the work of Hansch and others,[10] who have developed linear free energy relationships between biological activity and chemical parameters relating to partition, electronic, and steric factors. In order to minimise electronic and steric effects, we first considered

TABLE 1

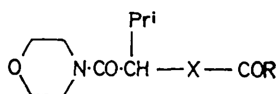
O[CH₂CH₂]₂N·CO·CH(R¹)·NH·CO·CH(R²)·CH₂·CO·NH·OH

Compound	R^1	R^2	Antibacterial activity *vs. S. aureus* and *E. coli*: *In vitro* MIC (μg/ml^{-1})		*In vivo/in vitro* (mice) (250 mg/kg^{-1} body wt.)	
			S. aureus	*E. coli*	*S. aureus*	*E. coli*
(1)	Pr^i	H	>100	>100	—	—
(2)	Pr^i	Me	250	>100	—	—
(3)	Pr^i	Et	32	>100	—	—
(4)	Pr^i	Pr^n	3·2	50	+	—
(5)	Pr^i	Bu^n	1·6	50	+	+
(6)	Pr^i	n-C_5H_{11}	1·6	12·5	+	+
(7)	Pr^i	n-C_6H_{13}	6·4	25	—	—
(8)	Pr^i	iso-C_5H_{11}	1·6	100	+	—
(9)	Pr^i	$[CH_2]_4CH$·	100	>100	—	—
(10)	Pr^i	Ph	100	>100	—	—
(11)	Pr^i	$PhCH_2$	2·0	>100	—	—
(12)	Pr^i	*p*-ClC_6H_4·CH_2	50	>100	—	—
(13)	Bu^i	n-C_5H_{11}	3·2	25	—	—
(14)	Me	n-C_5H_{11}	6·4	50	—	—
(15)	H	n-C_5H_{11}	25	64	±	—

a limited series of actinonin analogues with unbranched alkyl substituents (R^2 in Table 1), compounds (2)—(7),

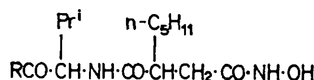
[9] J. Decombe, *Ann. Chim.* (*France*), 1932, **18**, 81.
[10] C. Hansch, *Accounts Chem. Res.*, 1969, **2**, 232; T. Higuchi and S. S. Davis, *J. Pharm. Sci.*, 1970, **59**, 1376.

TABLE 2



Compound	X	R	Antibacterial activity *vs. S. aureus* and *E. coli*: *In vitro* MIC (μg/ml^{-1}) *S.*	*E.*	*In vivo*/*in vitro* (mice) (250 mg kg^{-1} body wt.) *S.*	*E.*
(16)	$-NH{\cdot}CO{\cdot}CH(n\text{-}C_5H_{11})-$	NH·OH	50	>100	—	—
(17)	$-NH{\cdot}CO{\cdot}CH_2{\cdot}CH(n\text{-}C_5H_{11}){\cdot}CH_2-$	NH·OH	12·5	50	—	—
(18)	$-NH{\cdot}CO{\cdot}CH(n\text{-}C_5H_{11}){\cdot}CH_2{\cdot}CH_2-$	NH·OH	*	*	—	—
(19)	$-NH{\cdot}CO{\cdot}CMeEt{\cdot}CH_2-$	NH·OH	>100	>100	—	—
(20)	$-NH{\cdot}CO{\cdot}CHMe{\cdot}CHMe-$	NH·OH	>100	>100	—	—
(21)	$-CO{\cdot}NH{\cdot}CH(n\text{-}C_5H_{11}){\cdot}CH_2-$	NH·OH	>100	>100	—	—
(22)	$-NH{\cdot}CO{\cdot}CH(iso\text{-}C_5H_{11}){\cdot}CH_2-$	NH_2	>100	>100	—	—
(23)	$-NH{\cdot}CO{\cdot}CH(iso\text{-}C_5H_{11}){\cdot}CH_2-$	$NH{\cdot}NH_2$	>100	>100	—	—

TABLE 3

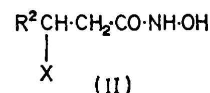


Compound	R	Antibacterial activity *vs. S. aureus* and *E. coli*: *In vitro* MIC (μg ml^{-1}) *S. aureus*	*E. coli*	*In vivo*/*in vitro* (mice) (250 mg/kg^{-1} body wt.) *S. aureus*	*E. coli*
	N, CH_2OH †	2	64	+	—
(24)	N	1·6	>100	+	—
(25)	N	0·8	25	+	—
(26)	N, CH_2OH	32	>100	+	—
(27)	N	0·8	>100	+	—
(28)	Me, O, N	0·4	25	+	+
(29)	Me, O, N, Me	1·6	50	+	—
(30)	N	0·8	25	+	—
(31)	N, NH	1·6	50	+	—
(32)]	O_2S, NH	>100	>100	—	—

* Insufficient material for accurate determination of MIC; — = inactive; ± = slightly active; + = active. † LLD-Isomer (actinonin).

and obtained a significant relationship between the *in vitro* activity against *S. aureus* and the partition constant π [equation (i), Table 4]. The lack of activity of compound (1) is in agreement with the equation. The parabolic relationship between activity and partition is consistent with passive transport of the drug substance through lipid membranes to the site of action.[11] The variation of activity due to substituents on the amino-acid unit (R^1 in Table 1) could also be explained in terms of partition, and the use of these compounds (13)—(15) in deriving the regression gave equation (ii) (Table 4) which is highly significant ($P < 0{\cdot}01$) and accounts for 95% of the variance. However, inclusion of branched chain compounds (8)—(12) in R^2 gave a very poor correlation, equation (iii), suggesting that branching in R^2 reduces activity. This may be quantified by comparison with the work of Newman *et al.*,[12] who studied the rate of acid-catalysed esterification of a series of aliphatic acids. This process, which involves a tetrahedral transition state, is largely insensitive to polar influences, and the rate differences are mainly due to steric factors.[10] Thus, if the actinonin molecule is considered as a substituted aliphatic acid derivative (II) with the steric effect of the morpholinovalyl fragment (X) taken as constant, the effect of branching in R^2 may be estimated by comparison of the rate constants for esterification of corresponding acids as determined by Newman.

The logarithm of this ratio may be regarded as a steric free energy term, ΔE_s, reflecting the change of E_s due to the effect of R^2 in this system. Inclusion of ΔE_s as a substituent constant gave a highly significant correlation for compounds (2)—(15) [equation (iv), Table 4].



Furthermore, the malonyl (16) and glutaryl (17) analogues may be considered in the same way leading to equation (v) (Table 4), which successfully correlates the activities of compounds (2)—(17) (Table 5). The inactivity of compounds (19) and (20) is consistent with equation (v) because α-branching and multiple β-branching should lead to high values of the steric term. It may be concluded that biological activity is associated with the formation of a tetrahedral transition state at the hydroxamic acid carbonyl carbon atom and that this may be hindered sterically by substituents on the α, β, and γ carbon atoms. The central amide linkage is also necessary however, since the compound (21) with this function reversed is inactive.

The effect of varying the heterocyclic component (Table 3) could not be evaluated quantitatively because the partition constants were not available for many of the substituents. However, the partition of many of the

[11] C. Hansch and J. M. Clayton, *J. Pharm. Sci.*, 1973, **62**, 1; S. H. Yalowsky and G. L. Flynn, *ibid.*, p. 210.

[12] M. S. Newman, 'Steric Effects in Organic Chemistry,' Chapman and Hall, London, 1956.

TABLE 4

Correlations of the *in vitro* activity against *S. aureus* of actinonin analogues

Equation	Compounds used in regression		N	R	s	F
(i)	(2)—(7)	$\log A = -1{\cdot}572 + 3{\cdot}588\pi - 0{\cdot}817\pi^2$	6	0·993	0·136	103·7 **
		(11·22) (9·139)				
(ii)	(2)—(7), (13)—(15)	$\log A = -1{\cdot}377 + 3{\cdot}189\pi - 0{\cdot}691\pi^2$	9	0·975	0·192	58·4 ***
		(8·409) (6·807)				
(iii)	(2)—(15)	$\log A = -1{\cdot}034 + 2{\cdot}457\pi - 0{\cdot}511\pi^2$	14	0·656	0·641	4·2 *
		(2·450) (2·071)				
(iv)	(2)—(15)	$\log A = -0{\cdot}930 + 3{\cdot}530\pi - 0{\cdot}787\pi^2 - 2{\cdot}421\Delta E_s$	14	0·968	0·225	49·2 ***
		(9·509) (8·533) (8·925)				
(v)	(2)—(17)	$\log A = -0{\cdot}933 + 3{\cdot}563\pi - 0{\cdot}799\pi^2 - 2{\cdot}254\Delta E_s$	16	0·958	0·239	45·0 ***
		(9·045) (8·255) (9·110)				

A = molecular weight/minimum inhibitory concentration, π = partition substituent constant, N = number of compounds used to derive the regression, R = correlation coefficient, s = standard deviation, F = variance ratio test statistic for the overall equation, $\Delta E_s = \log[k(MeCO_2H)/k(RCO_2H)]$ (see Table 5). Figures in parentheses are the Student t values for the individual coefficients of the equation. Asterisks indicate the probability by chance $^*p < 0{\cdot}05$ $^{**}p < 0{\cdot}01$ $^{***}p < 0{\cdot}001$.

most active compounds would not be expected to differ greatly from that of actinonin, whereas the least active compound (**32**) would be expected to differ quite considerably by virtue of the highly hydrophilic sulphone group. It is probable therefore that variation of activity with the heterocyclic group is partition dependent.

TABLE 5

In vitro activity against *S. aureus* correlated with chemical parameters [equation (v)]

Compound	MIC	π *	R in corresponding acid RCO_2H	$\frac{k_{40}(MeCO_2H)}{k_{40}(RCO_2H)}$	log(M/MIC) Obs.	Calc.	Diff.
(2)	250	0·5	Pr^n	2·02	0·110	−0·099	0·209
(3)	32	1·0	Bu^n	2·02	1·013	1·083	0·070
(4)	3·2	1·5	Bu^n	2·02	2·031	1·865	0·166
(5)	1·6	2·0	Bu^n	2·02	2·349	2·248	0·101
(6)	1·6	2·5	Bu^n	2·02	2·366	2·231	0·135
(7)	6·4	3·0	Bu^n	2·02	1·780	1·814	0·034
(8)	1·6	2·3	Bu^n	2·02	2·366	2·286	0·080
(9)	100	1·75	iso-C_5H_{11}	9·6	0·568	0·581	0·013
(10)	100	2·2	iso-C_5H_{11}	9·6	0·577	0·763	0·186
(11)	2·0	2·7	$Ph[CH_2]_2$	2·27 ‡	2·292	1·997	0·148
(12)	50	3·4	$Ph[CH_2]_3$	2·27 ‡	0·930	1·078	0·148
(13)	3·2	1·7	Bu^n	2·02	1·730	2·066	0·336
(14)	6·4	3·0	Bu^n	2·02	2·081	1·814	0·267
(15)	25	1·2	Bu^n	2·02	1·120	1·444	0·324
(16)	50	2·0	Bu^s	10·1	0·854	0·672	0·182
(17)	12·5	3·0	Bu^n	2·02	1·489	1·814	0·325

* C. Hansch, J. E. Quinlan, and G. L. Lawrence, *J. Org. Chem.*, 1968, **33**, 347. † Ref. 12; ΔE_s is the logarithm of this parameter. ‡ Calc. from data of J. J. Sudborough and J. M. Gittins (*J. Chem. Soc.*, 1909, **95**, 315).

The *in vivo*/*in vitro* activity measurements against *S. aureus* indicate that only those compounds with a low MIC showed activity. Many of the compounds showing activity *in vivo*/*in vitro* were examined *in vivo* (mice) but no activity was observed.

The activity against *E. coli* is lower than against *S. aureus* and, in general, structure–activity relationships appear similar although the biological data are not adequate for quantitative treatment. No *in vivo* activity was observed.

The actinonin molecule is known to undergo cleavage readily at the central amide bond by a mechanism which possibly involves intramolecular assistance by the protonated hydroxamic acid residue.[3] The lack of *in vivo* activity of this series is therefore almost certainly due to metabolism to inactive components. The most active compound (6) in the series in the *in vivo*/*in vitro* test appeared to be appreciably more stable to acidic hydrolysis than actinonin itself.

We thank Dr. K. Bowden (University of Essex) for discussions.

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