PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Structural Requirements for Antibacterial Activity and <latex>β</latex>-Lactamase Stability of 7<latex>β</latex>-Arylmalonylamino-7<latex>α</latex>-Methoxy-1-Oxacephems

T. Yoshida

Phil. Trans. R. Soc. Lond. B 1980 **289**, 231-237
doi: 10.1098/rstb.1980.0041

Phil. Trans. R. Soc. Lond. B **289**, 231–237 (1980)
Printed in Great Britain

Structural requirements for antibacterial activity and β-lactamase stability of 7β-arylmalonylamino-7α-methoxy-1-oxacephems

By T. Yoshida
Shionogi Research Laboratory, Shionogi & Co., Ltd, Fukushima-ku, Osaka 553, *Japan*

Replacement of a sulphur atom by an oxygen at the 1-position of the cephem nucleus generally resulted in fourfold to sixteenfold increase of antibacterial activity in each pair of the structural congeners. However, the increased antibacterial activity caused by the replacement was accompanied by instability to β-lactamase to some extent, which was due presumably to the increased chemical reactivity of the β-lactam ring system. The aim of the research effort is to confer β-lactamase stability and expand the Gram-negative spectrum. Two types of substituents have been demonstrated to protect 1-oxacephem from enzymic hydrolysis and their protecting effects were specifically related to the types of β-lactamases derived from Gram-negative bacteria: the 7β-malonylamino function is specific to cephalosporinase and the 7α-methoxy group to penicillinase. The complementary effect of these substituents was clearly demonstrated. This line of studies led us to prepare the clinical candidate 6059-S, which possessed widely expanded antibacterial spectra against Gram-negative bacteria including indole-positive *Proteus*, *Enterobacter*, *Sarratia marcescens*, *Pseudomonas aeruginosa* and *Bacteroides fragilis*.

Introduction

Penicillin and cephalosporin have been attractive agents and the most reliable materials for modification because of an excellent selectivity of biological action. In 1970, 2-thiacephem system was prepared by the Woodward group and showed antibacterial activity (Heusler 1972). Since then a number of structural modifications in the cephem nucleus have appeared in the literature. More recently, new β-lactam antibiotics with different ring systems, such as thienamycin (Stapley *et al.* 1979) and clavulanic acid (Reading & Cole 1977), have been isolated from the natural sources. Although none of them has taken the place of the cephalosporins and penicillins currently used in clinics, this line of progress encourages us to expect coming the era of new β-lactam as a useful medicine.

The synthesis of 1-oxacephems was first reported from two laboratories (Wolfe *et al.* 1974; Cama & Christensen 1974). The latter investigators have suggested that the *in vitro* antibacterial activity of cephalosporins were not greatly improved by replacing sulphur by oxygen in the nucleus (Cama & Christensen 1974; Firestone *et al.* 1977).

We have extensively investigated the biological properties of the 1-oxacephem derivatives in comparison with the 1-sulphur congeners, and found a distinct difference between them in antibacterial activity (Narisada *et al.* 1977).

Antibacterial shift by S → O replacement of the cephem nucleus

At the beginning of our studies, a number of 1-oxa congeners of 3-methylcephalosporins were prepared with a variety of side chains at the 7β-position (Narisada *et al.* 1977). The Gram-positive and Gram-negative antibacterial activity of 3-methylcephalosporin are located in

figure 1 with dots in the coordinates, and open circles indicate those of the 1-oxa congeners. A change of antibacterial activity caused by the S → O replacement is shown by an arrow. So, the greater is the distance between the two points, the greater the difference in activity. Antibacterial shift is always 4–16 times better in the 1-oxacephem analogues, although there is one exception, a phenylglycyl derivative, which had totally lost any activity by the S → O replacement. This exceptional decrease in the antibacterial shift might be due to nucleophilic attack by the α-amino group on the β-lactam (Indelicato *et al.* 1974).

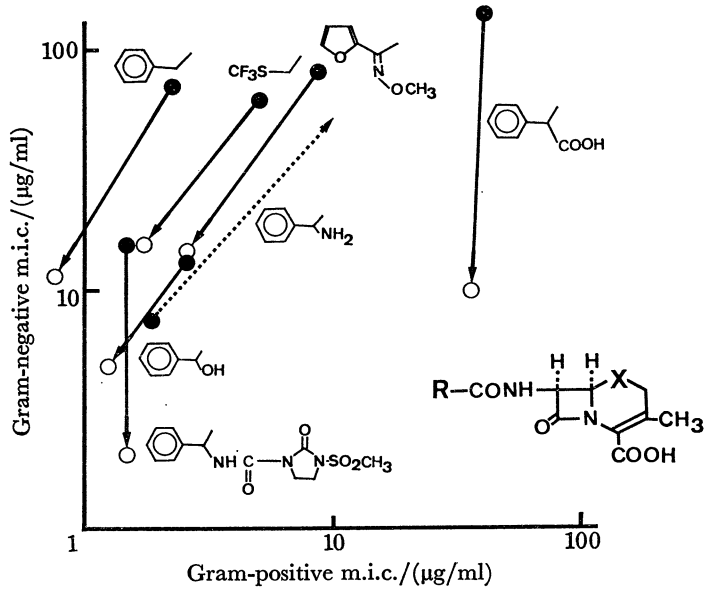


FIGURE 1. Comparative antibacterial activity of 3-methyl-1-oxacephems (○) and the 1-sulphur congeners (●) with various side chain (R) modifications shown beside each of the points. The antibacterial shift between each pair of the congeners is indicated by an arrow. The minimum inhibitory concentration (m.i.c.) for each compound was determined by the agar dilution method. The points represent the geometrical means resulting from five strains of Gram-positive bacteria and five strains of Gram-negative bacteria.

A degree of the shift appears to depend on the type of side chain structure. A phenylmalonyl derivative manifested the greatest shift (almost 16-fold increase) by the S → O replacement so far found. In general, antibacterial shift seems to be greater in Gram-negative activity than in Gram-positive activity.

The malonylamino derivative of 1-oxacephem was qualified for further modification from the following observations: (1) the greatest shift by S → O replacement, (2) a broad spectrum to Gram-negative bacteria and (3) the shift generally favoured Gram-negative activity.

Then modification at C-3′ was made for 7β-phenylmalonylamino-1-oxacephems. The superiority of the thio-substituted compounds is quite evident and those are shown in figure 2. The tetrazolylthiomethyl compounds are best in activity against Gram-positive and Gram-negative pathogens, that is approximately 100 times as active as the non-substituted parent.

The antibacterial shift caused by the S → O replacement of the malonylamino derivative was carefully examined by using 17 strains of Gram-negative bacteria, which included *E. coli*, *Klebsiella*, *Proteus* (indole-positive and indole-negative), *Enterobacter*, *Serratia marcescens* and *Pseudomonas aeruginosa* (figure 3).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

From these detailed analyses of m.i.c. correlation between each pair of congeners, the 1-oxa analogue still showed 16-fold greater activity than 1-sulphur congener against the majority of the tested organisms, including *Serratia* and *Enterobacter*. Against *Proteus*, the shift is slightly smaller but still a fourfold increase.

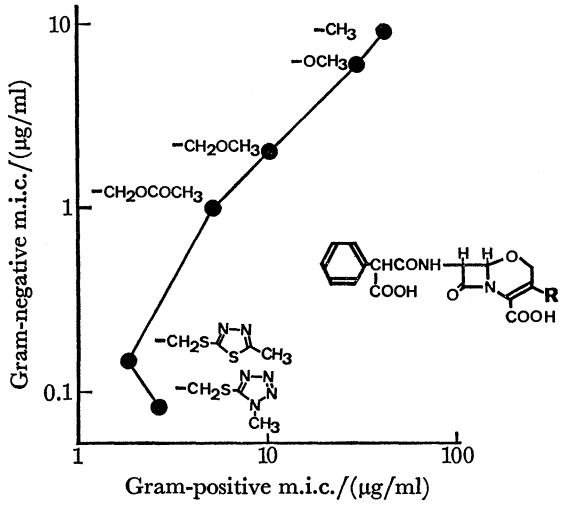


FIGURE 2. Antibacterial shift of 7β-phenylmalonylamino-1-oxacephems by modification at the C-3′ position. The substituents (R) are shown beside each point. The points represent the m.i.cs as described in figure 1.

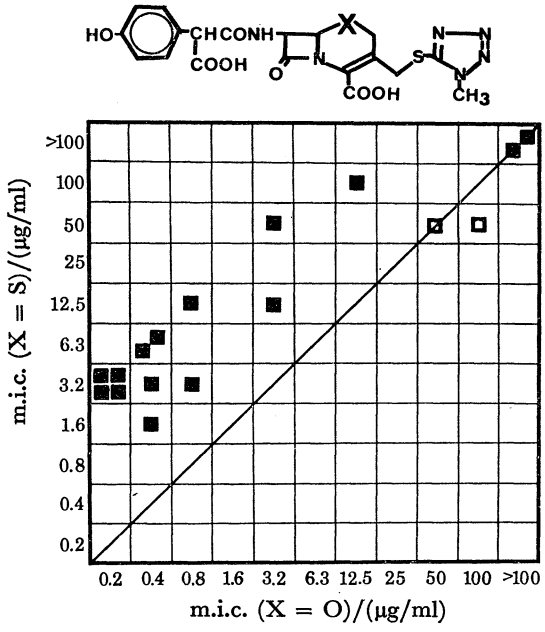


FIGURE 3. Correlation of m.i.cs between 7β-*p*-hydroxyphenylmalonylamino-1-oxacephem and the corresponding 1-sulphur congener against β-lactamase-producing (□) and non-producing (■) Gram-negative bacteria.

On the contrary, in β-lactamase-producing strains of *E. coli* and *Klebsiella*, illustrated by open square in figure 3, virtually no shift was demonstrated and one of them appears to be even less sensitive to the 1-oxa analogue than to the 1-sulphur congener.

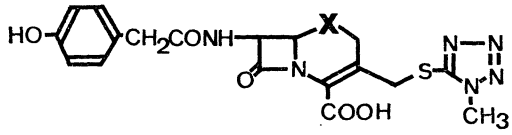
These observations suggest that decreased antibacterial shift to resistant strains is due to reduced stability to β-lactamases. To test this possibility, the relative rate of hydrolysis was

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

measured by a photometric assay and compared between the pair of analogues by using various classes of β-lactamase from Gram-negative bacteria (table 1). A striking difference was demonstrated in all the β-lactamases tested. The relative V_{max} values of the 1-oxacephem were considerably higher than those of the 1-sulphur congeners. In the penicillinase type, the physiological efficiency was also consistent with the V_{max} data.

TABLE 1. RELATIVE RATES OF HYDROLYSIS OF 1-OXACEPHEM AND THE 1-SULPHUR CONGENER

(The rates of hydrolysis were determined by the spectrophotometric (u.v.) assay as described by Ross *et al.* (1973). V_{max} is expressed as the hydrolysis rate relative to cephaloridine (100). K_m is determined by calculating the reciprocal of the slope in a Lineweaver–Burk plot. Each of the enzyme preparations was partly purified by column chromatography. The β-lactamase was classified according to the proposal by Richmond & Sykes (1973).)



β-lactamase	class	X =	V_{max} S	V_{max} O	V_{max}/K_m S	V_{max}/K_m O
E. coli 6	Ib		80	840	1.36	1.65
E. cloacae 214	Ia		31	86	0.65	0.56
P. vulgaris 31	Ic		380	3600	2.23	2.25
E. coli W3110 R_{TEM}	III		13	240	0.05	0.86
E. cloacae 53	IV		7	56	0.22	4.67
Klebsiella sp. 363	IV		97	690	0.66	2.88

It is apparent from these results, obtained in antibacterial shift and β-lactamase sensitivity, that an oxygen replacement at the 1-position of cephem does significantly increase acylating ability. A possible explanation might be the strained β-lactam ring system according to Morin's prediction (1969). This was supported by the finding that the infrared frequency of β-lactam of the 1-oxacephem is 1778 cm^{-1}, which is 5 cm^{-1} higher than that of the sulphur congener.

As it was predicted that the penicillin-sensitive enzyme was inhibited by forming a thiol ester of β-lactam carbonyl and the active sites in enzyme protein (Lawrence & Strominger 1970; Spratt 1977), the higher acylating power of 1-oxacephem was thought to favour the greater activity against microorganisms.

SUBSTITUENT EFFECT ON β-LACTAMASE STABILITY

The research effort has been concentrated to confer β-lactamase stability without quenching the antibacterial shift. Two types of substituents have been demonstrated to protect 1-oxacephem from enzymic hydrolysis. Figure 4 illustrates the relative rate of hydrolysis of the differently substituted compounds by the variety of β-lactamases, which included species-specific cephalosporinase, namely class I enzyme according to Richmond's classification (Richmond & Sykes 1973) and penicillinase, e.g. classes V (RGN 238), III (R_{TEM}) and IV. All of the enzyme preparations were partly purified by column chromatography.

A non-substituted compound was equally hydrolysed by either type of β-lactamases. A 7α-methoxy substituent provides the resistance to destruction by penicillinase-type enzyme, while it has little effect on cephalosporinase-type. On the other hand, an incorporation of a carboxyl

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

function at the α-position of the side chain, e.g. a phenylmalonyl moiety, stabilizes 1-oxacephem against cephalosporinase-type enzymes, although there is only a negligible protecting effect from penicillinase-type enzyme. *Proteus vulgaris* enzyme always showed a different pattern from the other class I β-lactamases and behaved as a penicillinase-type. It is evident that the protecting effect of these two substituents was significantly related to the type of β-lactamase.

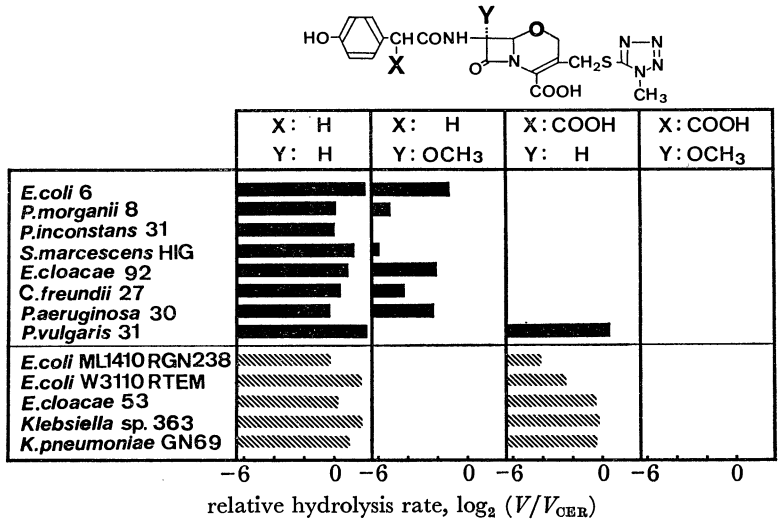


FIGURE 4. Effect of substituents, carboxyl group of side chain (X) or methoxyl group at the 7α-position (Y) or both upon the β-lactamase stability. The rate of hydrolysis (V) was determined by the microbiological assay with the use of *E. coli* B as described by O'Callaghan *et al.* (1969). The relative hydrolysis rate is expressed as the logarithm of the ratio between the hydrolysis rate of sample and that of cephaloridine (CER), and is indicated by a solid bar for cephalosporinase and a hatched bar for penicillinase.

When the 1-oxacephem compound was substituted by both functional groups, complete protection was demonstrated and none of the β-lactamases was able to hydrolyse it to any detectable extent. From the data given, it is postulated that both substituents strictly have a complementary effect on β-lactamase stability.

SUBSTITUENT EFFECT ON ANTIBACTERIAL ACTIVITY

The question has been raised as to whether or not such a highly stable doubly substituted compound tends to decrease antibacterial activity. Comparison of agar dilution m.i.c. values of these analogues clearly demonstrates the same complementary effect on antibacterial activity (table 2).

The unsubstituted analogue has very poor activity toward each of the tested strains. The sensitivity to penicillinase producing *E. coli* and *Klebsiella* was restored by a 7α-methoxy group but not by an arylmalonyl side chain. The same was true of *P. vulgaris* though the enzyme type produced was exceptional, as mentioned before. On the other hand, *Enterobacter* and *Serratia* produce inducible cephalosporinase and are specifically sensitive to the arylmalonyl derivative, but they appear to be rather refractory to the 7α-methoxy congener.

The doubly substituted analogue completely restored sensitivity to all of the tested organisms including *Pseudomonas*. The m.i.c. values were demonstrated to be the least among the four

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

derivatives. It is concluded that two substituents are unequivocally essential to the 1-oxacephem derivatives in respect of widening antibacterial spectrum and increasing Gram-negative activity.

Finally, this doubly substituted compound, which was designated as 6059-S, was selected as a clinical candidate. The agar dilution m.i.c. distribution of 6059-S was measured against various pathogenic bacteria (954 strains) recently isolated in domestic hospitals (figure 5). The antibacterial spectrum is extraordinarily expanded and includes the bacterial species normally refractory to the cephalosporins.

TABLE 2. EFFECT OF SUBSTITUENTS OF 1-OXACEPHEM ON ANTIBACTERIAL ACTIVITY

(The minimum inhibitory concentration was determined by the agar dilution method. β-Lactamase-producing strains are indicated by R in parentheses. The substituents X and Y are on the same chemical structure as described in figure 4.)

	m.i.c./(μg/ml)			
X =	H	H	COOH	COOH
Y =	H	OCH_3	H	OCH_3
E. coli	0.8	0.1	0.2	0.2
E. coli (R)	> 100	0.8	50	0.4
Klebsiella sp. (R)	> 100	0.2	100	0.1
P. vulgaris (R)	> 100	1.6	12.5	0.4
E. cloacae	> 100	100	0.4	0.2
S. marcescens	> 100	6.3	0.8	0.4
P. aeruginosa	> 100	> 100	> 100	12.5

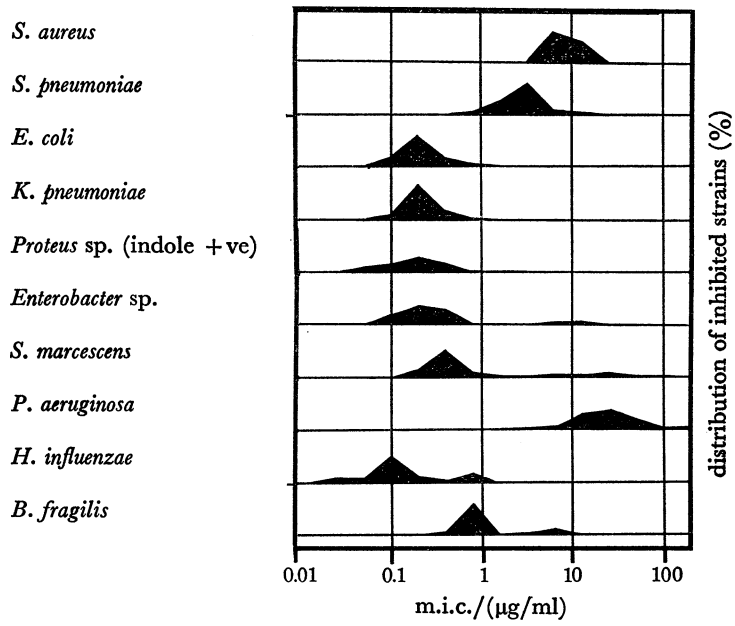


FIGURE 5. Antibacterial activity of 6059-S against 954 strains of clinical isolates of pathogenic bacteria. The minimum inhibitory concentrations was determined by the agar dilution method. The height of each column indicates 100% for each bacterial species tested.

Gram-negative pathogens, including the anaerobe *B. fragilis*, are equally inhibited by a concentration less than 1 μg/ml. As 70% of all the tested strains are resistant to aminobenzylpenicillin, it is evident that the activity of 6059-S is not affected by the presence of β-lactamase.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

I am extremely grateful to many colleagues in the Shionogi Research Laboratory for helpful critisism and discussion, particularly Dr W. Nagata, Dr M. Narisada and Mr K. Murakami. Thanks are also due to Dr H. Otsuka, the managing director of this laboratory, for his encouragement throughout this work. The technical assistance of Mr K. Motokawa and Mr K. Takasuka is recorded with appreciation.

References (Yoshida)

Cama, L. D. & Christensen, B. G. 1974 *J. Am. chem. Soc.* **96**, 7582–7584.
Firestone, R. A., Fahey, J. L., Maciejewicz, N. C., Petel, G. S. & Christensen, B. G. 1977 *J. med. Chem.* **20**, 551–556.
Heusler, K. 1972 In *Cephalosporin and penicillins: chemistry and biology* (ed. E. H. Flynn), pp. 276. N.Y.: Academic Press.
Indelicato, J. M., Norvilas, T. T., Pfeiffer, R. R., Wheeler, W. J. & Wilham, W. L. 1974 *J. med. Chem.* **17**, 523–527.
Lawrence, P. J. & Strominger, J. L. 1970 *J. biol. Chem.* **245**, 3653–3659.
Morin, R. B., Jackson, B. G., Mueller, R. A., Lavagnino, E. R., Scanlon, W. B. & Andrews, S. L. 1969 *J. Am. chem. Soc.* **91**, 1401–1407.
Narisada, M., Onoue, H. & Nagata, W. 1977 *Heterocycles* **7**, 839–849.
O'Callaghan, C. H., Muggleton, P. W. & Ross, G. W. 1969 *Antimicrob. Agents Chemother. 1968*, pp. 57–63.
Reading, C. & Cole, M. 1977 *Antimicrob. Agents Chemother.* **11**, 852–857.
Richmond, M. H. & Sykes, R. B. 1973 *Adv. microb. Physiol.* **9**, 31–88.
Ross, G. W., Chanter, K. V., Harris, A. M., Kirby, S. M., Marshall, M. J. & O'Callaghan, C. H. 1973 *Analyt. Biochem.* **54**, 9–16.
Spratt, B. G. 1977 *Eur. J. Biochem.* **72**, 341–352.
Stapley, E. O., Miller, T. W., Miller, A. K., Hendlin, D., Mochales, S., Hernandez, S., Woodruff, W. B. & Birnbaum, J. 1979 *J. Antibiot.* **32**, 1–12.
Wolfe, S., Ducep, J. B., Tin, K. C. & Lee, S. L. 1974 *Can. J. Chem.* **52**, 3996–3999.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES