Polyoxotungstates Reduce the β-Lactam Resistance of Methicillin-Resistant *Staphylococcus aureus*

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Abstract: Bacterial strains isolated from clinical specimens have become more and more resistant to many antimicrobials. This is because we have consumed large amounts of strong antimicrobials over long periods of time and thus bacterial cells are able to survive by altering the target(s) of antimicrobial agents. A good example of this phenomenon is methicillin-resistant *Staphylococcus aureus* (MRSA). One of the cell wall-synthesizing enzymes (known as PBP2') of this pathogen has low affinity to β-lactams, and therefore the bacterial cells continue to grow even under high concentrations of the agents. However, this drug resistance does not seem to be total. They seem to have some weak spots and several substances are known to sensitize strains of MRSA to β-lactams. This review discusses the ability of polyoxotungstates (POTs) to sensitize MRSA to β-lactams by reducing the expression of PBP2'. It is also possible that the sensitization is a type of stress response of MRSA to POTs. This idea may provide a hint for the development of a new antimicrobial agent.

Keywords: methicillin-resistant *Staphylococcus aureus*, polyoxotungstate, β-lactam antibiotics, penicillin-binding protein.

INTRODUCTION

When a patient suffers from a bacterial infection, physicians usually administer effective antimicrobials to the patient. All the antibacterial agents introduced in clinical use are potent inhibitors of certain essential reactions of the bacterial cells. For instance, aminoglycosides [1, 2], tetracyclines [3, 4] and macrolides [3, 5] suppress the bacterial growth by inhibiting protein synthesis. Quinolones are inhibitors of DNA gyrase and topoisomerase, both of which are essential enzymes for DNA replication [6, 7]. β-lactam antibiotics directly inhibit several cell wall-synthesizing enzymes (known as penicillin-binding proteins (PBPs)), thereby killing the bacterial cells [8-10].

Unfortunately, many kinds of bacterial strains have rapidly developed several types of antimicrobial resistance, and the following four mechanisms are known as major pathways, whereby bacterial strains escape the antibacterial action [11]:

1. Acquisition of an enzyme modifying the chemical structure of antimicrobials. For instance, β-lactamase hydrolyzes the β-lactam ring of β-lactams and inactivates the agents [8-10]. Therefore, bacterial strains that have acquired this enzyme will become resistant to β-lactams unless the molecular structure of the agent is specially designed so as not to be hydrolyzed by the enzyme (*e.g.*, methicillin (dimethoxyphenylpenicillin, DMPPC)) [8, 9], or inhibitors of the enzyme (*e.g.*, clavulanic acid) are combined with the agent [8, 12].
2. Escaping from the action of the agents. This is further classified into the following two subgroups: (a) overproduction of the essential factors for bacterial growth, which are the targets of the antimicrobials (quantitative escaping), and (b) lowering affinity of the target molecule to the agents (qualitative escaping). A good example of category (a) is bacterial resistance to sulfonamides [6, 11, 13]. These agents are inhibitors of the folic acid metabolism, but the metabolic activity of some strains with resistance increases so high that it can not be inhibited by therapeutic concentrations of the agent. Thus, in category (a), the cells of the resistant strain can continue to grow. An example of category (b) is discussed in the next section.
3. Lowering the penetration of antimicrobials into bacterial cells. Resistant strains in this category are frequently isolated in Gram-negative bacterial species rather than Gram-positive ones. This is because Gram-negative bacteria have an outer membrane outside the cell wall, which limits various substances from incorporating into the cells [8, 11]. Many protein molecules are located in the outer membrane and some of them act as a "gate" (known as a porin) to many substances in the culture medium. It is well known that an alteration and/or mutation in the porin protein will result in multi-drug resistance [11].
4. Pumping antimicrobials out from bacterial cells. This pump is considered a type of excretion system of the bacterial cells, which excretes many kinds of antimicrobials [11]. Therefore, an acquisition and/or alteration (due to mutation) of the pumping protein will also result in multi-drug resistance. Interestingly, a similar mechanism is found in cancer cells having resistance to anticancer agents [14].

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THE HISTORY OF β-LACTAM RESISTANCE IN *STAPHYLOCOCCUS AUREUS*

Formerly, the main mode of the antimicrobial resistance was "acquisition of an enzyme modifying the chemical structure of antimicrobials" (see "1" in the former section). This is because the gene encoding such an enzyme is often located on a plasmid, and thus this type of drug resistance is easily transmittable to other bacterial strains.

However, current trends in antimicrobial resistance have gradually shifted to be "escaping from the action of the agents" (see "2" in the former section). This is because recent advances in pharmacological and chemical techniques have allowed the molecular improvements of several antimicrobials so as not to be decomposed by a modifying enzyme. Therefore, bacterial cells could not survive any more by simply getting a gene of a modifying enzyme, and they have to completely escape from the action of these refined agents. This is a risky way for bacterial cells, since major gene alteration (*e.g.*, rearrangement, mutation) is usually required to "escape" the action of antimicrobials, and thus every bacterial strain can not always win this "stake". However, there seems to be no other way to overcome the "hardships" from humans, and methicillin-resistant *Staphylococcus aureus* (MRSA) is a good example of this phenomenon. This review will focus on this pathogen having a unique and interesting mechanism of resistance.

S.aureus is one of the major and global causative pathogens in humans. This type of bacteria frequently causes serious life-threatening problems especially in hospitalized patients in immuno-compromized states [15]. Clinical introduction of penicillin G (PCG) in the 1950s was a "godsend", and indeed, PCG was very effective at that time [8, 9]. However, PCG became ineffective soon after, because many strains of *S.aureus* acquired a plasmid carrying the gene of penicillinase (PCase), which is known as a type of β-lactamase (see "1" in the former section), yielding resistance.

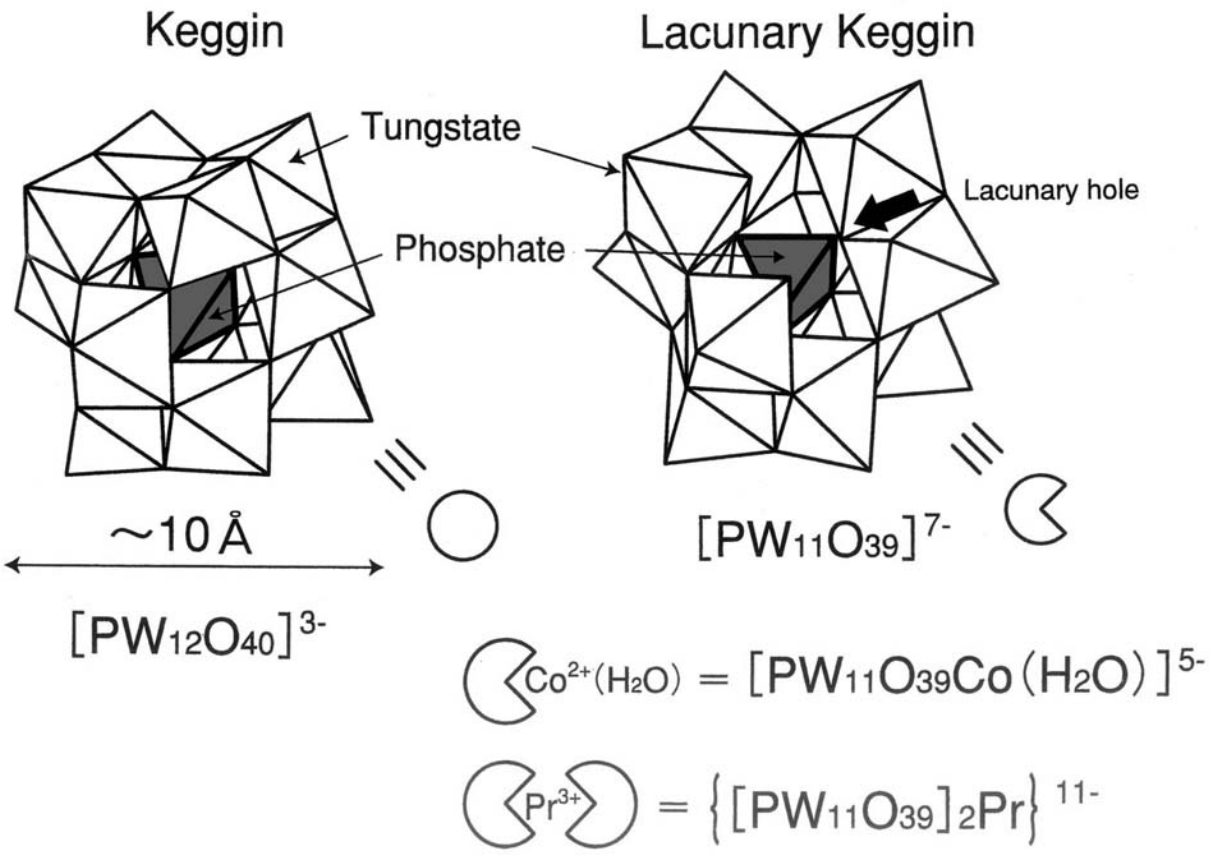

Fig. (1). Structure of "Keggin"-structural POTs.

The left panel is the structure of the PW_{12} molecule ($[PW_{12}O_{40}]^{3-}$). One phosphate (PO_4^{3-}) unit, which is located in the center of the molecule (shaded), is surrounded by twelve tungstate (WO_4^{2-}) units. This is a typical example of a "Keggin"-structural POT, which is the simplest and the most familiar POT. In addition to this type, many other kinds of POTs are known to date, including "Anderson"- and "Dawson"-structural POTs [27-30, 46, 47].

The right panel shows the structure of PW_{11} molecule ($[PW_{11}O_{39}]^{7-}$). One tungstate unit is released from the PW_{12} molecule by partial hydrolysis, and this site is known as a "lacunary hole". This type of compound is sometimes known as a "mono-vacant" lacunary-Keggin. Some metal cations such as cobalt ions (Co^{2+}) are trapped by the lacunary hole and then forming a new compound called "lacunary-substituted" POT [48]. Lanthanide elements (such as praseodymium, Pr) give a "2:1" complex, and this structure is called "sandwich Keggin" [48]. If two molecules of "Keggin" species are polymerized without sandwiching any material, this is called a "double Keggin". "Dawson" is a type of dimer of the "tri-vacant lacunary-Keggin".

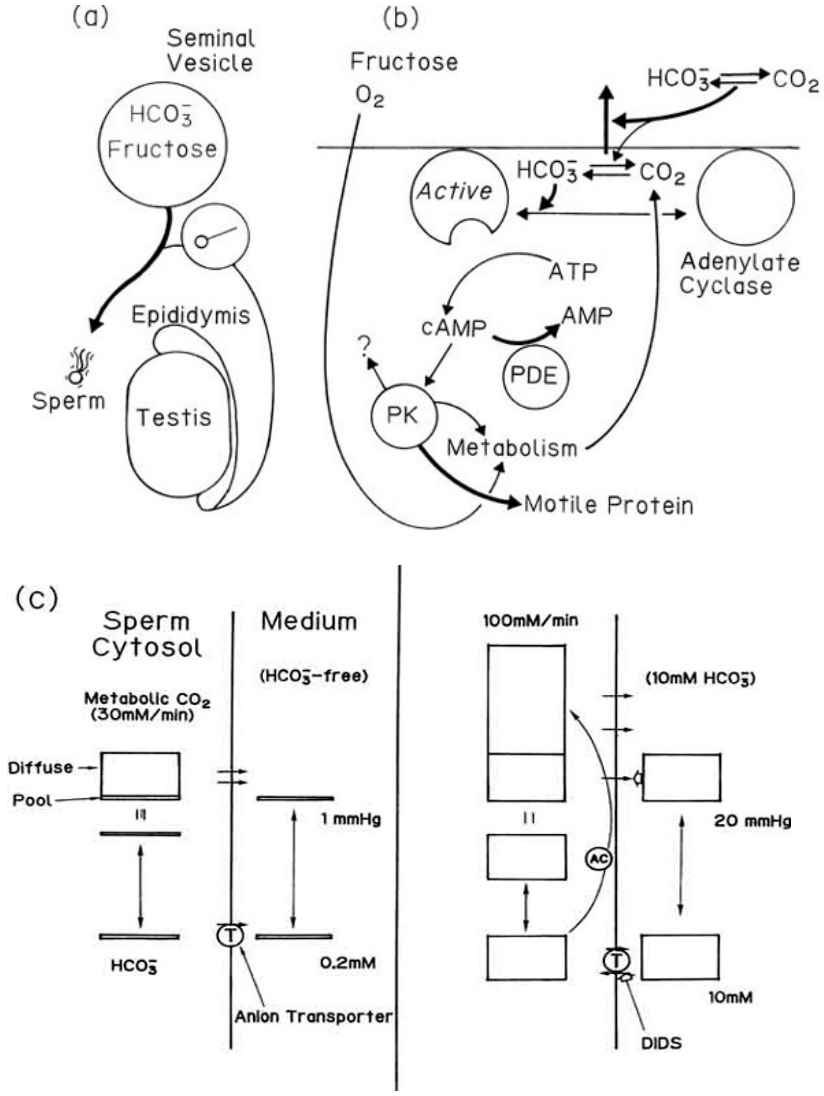

Fig. (2). The discovery of the biological effects of POTs.

(a) Most mammalian sperm cells stored in the cauda epididymis are immotile, yet already matured, and interestingly, they become rapidly activated during the short period of ejaculation [138, 139]. Formerly, this mechanism was quite unknown, but we found a "factor" activating the sperm cells in the seminal vesicle fluid [140]. Later, this factor was purified and identified as bicarbonate (HCO_3^-) [33]. At ejaculation, the "quiescent" sperm cells stored in the cauda epididymis first come into contact with a high concentration of HCO_3^-, thereby being activated rapidly.

(b) It was generally accepted that cAMP was the essential factor of the activation of sperm [141, 142]. However, it was quite unknown at that time what activates the adenylate cyclase (AC) of the sperm and produces cAMP, since this enzyme is insensitive to various stimulants established in that of the somatic cells [143, 144]. Some investigators thought, rather, that a certain regulatory molecule of phosphodiesterase (PDE), which decomposes cAMP before activating protein kinase (PK), may play a more important role, since the activity of PDE is 100 times higher than that of AC and no direct activator of the sperm AC was found [145]. Later, we found that HCO_3^- directly activates the sperm AC [33] and concluded that the activation of the sperm at ejaculation is principally explained by an increase in intracellular cAMP level, which is induced by activation of the sperm AC with HCO_3^- in the seminal vesicle fluid [31].

(c) Initially, we had thought that extracellularly added HCO_3^- was transported into the cytosol, thereby activating the sperm AC. Therefore, it was expected that anion channel-blockers such as 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS) [34-37] inhibited the HCO_3^--induced activation of sperm, but the result was opposite (*i.e.*, DIDS further enhanced the HCO_3^--induced activation [32]). Curiously, PW_{11}/SiW_{11} showed the same effect as DIDS [32], and later, PW_{11}/SiW_{11} was found to have an inhibitory effect on the anion transport activity of the sperm cells and erythrocytes [32, 38]. That was the first discovery of the biological effects of POTs. The mechanism of this phenomenon is now considered as follows:

Sperm cells produce considerable amounts of metabolic CO_2, even before being activated [31], but most of the metabolic CO_2 diffuses rapidly from inside the sperm (left panel). When HCO_3^- is exogenously added to the sperm (right panel), this outside-directed CO_2 diffusion will be inhibited by a simple gas equilibrium. In such a condition, the intracellular HCO_3^- derived from the metabolic CO_2 rapidly increases enough to activate the sperm AC so that more CO_2 will be generated and pooled inside the sperm. When the anion channel is blocked, the endogenous HCO_3^- will accumulate inside the sperm cytosol. This promotes a stronger motile action of the sperm by further activation of the sperm AC [32].

Investigators in those days tried to solve this problem and succeeded in improving the chemical structure of PCG so that it would no longer be hydrolyzed by PCase. This first agent was DMPPC. DMPPC has a bulky side chain near the β-lactam ring that is not incorporated into the active site of PCase due to steric hindrance, and this idea (*i.e.*, molecular improvement against β-lactamases) has been successful until today (*e.g.*, the development of the new generation cephems) [8-10]. At that time, nobody had doubted the conquest of DMPPC over PCase-positive *S.aureus*. However, a resistant strain was isolated only one year after the clinical use of DMPPC: This was MRSA [16].

It is well accepted that the resistance of MRSA to β-lactams is not due to antibiotic-modifying enzymes such as PCase [8, 9, 17, 18]. Rather, it is principally attributed to the acquisition of an altered PBP [19], designated "PBP2'" or "PBP2a", which has lowered affinity to β-lactams [20-23]. From analysis of the PBP2'-encoding gene (*mecA*), PBP2' is now considered to be a type of chimera protein, which probably originated through gene fusion [24]. As a result, PBP2' shows very low affinity for β-lactams and is not inactivated by therapeutic concentrations of the agents [20-23]. Because of the residual activity of PBP2', these cells can make cell walls and continue to grow, whereas other PBPs are all inactivated by β-lactams [23, 25]. In addition to PBP2', strains of MRSA frequently acquire several factors increasing their resistance to β-lactams and other classes of antimicrobials [26]. This means that only a limited number of antimicrobials are effective for MRSA strains.

However, recent investigation has shown that the resistance of MRSA is neither invincible nor total. Several substances make strains of MRSA susceptible to β-lactams. One of these substances is undecatungstophosphate ($[PW_{11}O_{39}]^{7-}$, PW_{11}), a type of "Keggin"-structural polyoxotungstates (POTs) [27-30]. This matter will be discussed further in this review.

THE DISCOVERY OF THE BIOLOGICAL EFFECTS OF POTS

The molecular structure of PW_{11}, a representative POT [27-30], is shown in Fig. **1**. The discovery of the biological effects of POTs was made entirely by chance, and the details are summarized in the legend of Fig. **2**.

Briefly, it was found that PW_{11} (presented as "PTA" in our previous report) further enhanced the bicarbonate (HCO_3^-)-induced activation of the mammalian sperm by inhibiting anion transport activity. When the anion channel is inhibited, endogenous HCO_3^- derived from sperm metabolic CO_2 will accumulate inside the sperm cytosol [31, 32]. Since HCO_3^- directly activates the sperm adenylate cyclase [33], cAMP levels of the sperm cytosol further increase under such conditions and this promotes a stronger motile action of the sperm [32]. It is well known that there are positively charged amino acid residues in the center of anion channels, and they act as a "tout" for negatively charged anions [34-37]. Since PW_{11} is a polyanion, PW_{11} strongly interacts with the positively charged part of the anion channel, thereby inhibiting the anion transport activity (see also the "Appendices" section) [38].

As shown in Fig. **1**, PW_{11} is a heptabasic anion with a molecular diameter of approximately 10Å [27, 30]. The high anionic valence present in such a small area is responsible for the strong charge interaction between PW_{11} and its target molecule(s), which is hardly affected by steric hindrance. For instance, our previous studies demonstrated that the salting-in effect and elution power in affinity chromatography of PW_{11} were 100~1000 fold higher than those of NaCl [38-40]. It is now considered that such compounds may be useful as anionic probes, which will affect certain biochemical and/or biological systems where charge interaction plays an important role [41]. The other properties of tungsten compounds are described in the "Appendices" section.

THE DISCOVERY OF SENSITIZING EFFECTS OF POTS ON MRSA

The sensitizing effects of POTs on MRSA (Fig. **3**) were also found entirely by chance [42-44]. Briefly, a mixture of tungstate (WO_4^{2-}) and phosphate (HPO_4^{2-}), which was prepared for another purpose and left sitting for a long period, was spilled by accident on an agar plate, wherein antimicrobial susceptibility of an MRSA strain was being measured by the paper disk method. When the growth inhibition zone spread out, its boundary coincided exactly with the mark of the spilt solution. We initially considered this to be due to a direct killing effect of the solution, since WO_4^{2-} is a heavy metal compound. However, MRSA was able to grow normally on the mark farther away from disks containing β-lactam antibiotics, and also on the mark around disks of other types of antibiotics. This meant that the solution enhanced the antibacterial effect of β-lactams rather than directly killing the cells alone. We then considered that a certain factor (named "Factor *T*" after "*T*ungstate") was formed in the aged solution during storage, because a freshly prepared mixture of WO_4^{2-} and HPO_4^{2-} had no effect [42].

Subsequently, this factor was purified and identified as PW_{11} [45], and it was also found that many POTs have similar sensitizing effects on MRSA [46, 47]. Of the related tungsten compounds tested, Keggin-structural POTs (*e.g.*, $[PTi_2W_{10}O_{40}]^{7-}$) and their mono- or tri-vacant lacunary species (*e.g.*, $[SiW_{11}O_{39}]^{8-}$ (undecatungstosilicate, SiW_{11})) are found to have the strongest sensitizing effects [46-49]. Although these compounds alone do not show significant antibacterial activity unless an excess amount (~1mM) is added, minimum inhibitory concentrations (MICs) of various β-lactams against MRSA are reduced by 1/10~1/1000 in the presence of POTs, whereas their constituents (*e.g.*, WO_4^{2-}, HPO_4^{2-}) have no effect [42, 43, 45-49]. Furthermore, there is no synergism of POTs with other classes of antibiotics (Fig. **3**), and POTs have no effect against bacterial strains other than staphylococcal species [42, 43, 45, 49].

On the other hand, it is well known that molybdenum (Mo) and vanadium (V) can form polyanionic compounds having a molecular structure and charge similar to POTs [30]. However, these polyanionic compounds containing Mo and/or V are not so effective when compared to POTs [47]. The reason is unclear, and can not be simply explained by the pH-stability of the compounds.

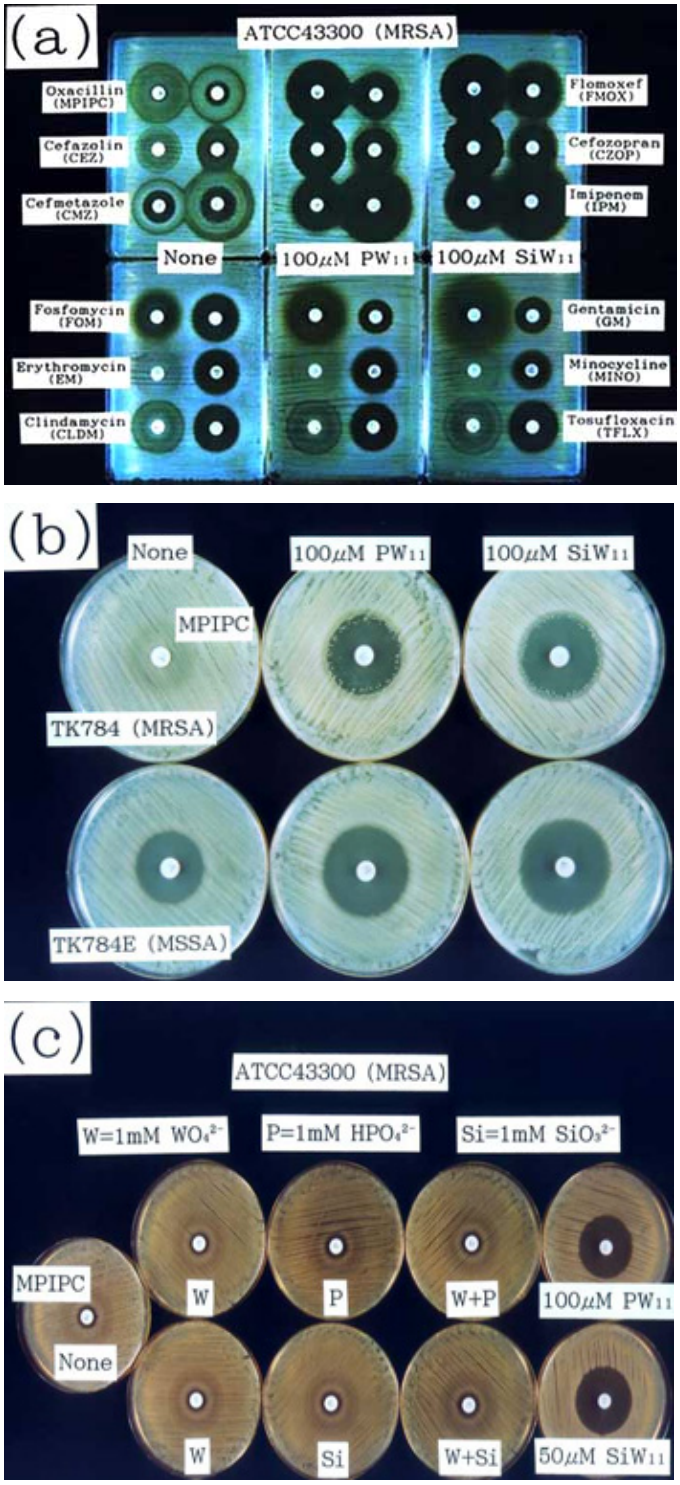

Fig. (3). Effects of POTs on MRSA strains.

The sensitizing effects of POTs can be easily observed using the paper disk method. The experimental details are described in our previous reports [42-49].

(a) PW_{11}/SiW_{11} sensitized MRSA strains only to β-lactam antibiotics. This is because PW_{11}/SiW_{11} reduced the expression of PBPs, known to be targets of β-lactams.

(b) PW_{11}/SiW_{11} had a weak sensitizing effect on a methicillin-susceptible *S.aureus* (MSSA) strain, but this was not obvious because oxacillin (methylphenylisoxazolylpenicillin, MPIPC) had already acted strongly on this strain. TK784E is a sensitized mutant which was made by eliminating the *mecA* gene from the parent strain TK784 (*i.e.*, both strains are genetically identical except for methicillin-resistance) [23].

(c) The constituents of POTs (*e.g.*, HPO_4^{2-}, WO_4^{2-}) have almost no effect.

Table 1. The Effect of Various Compounds on Strains of *S. aureus*

Compounds	Concentration	Stress Promoter[a]	Fold Induction (%)[b]	Enhancement Index[c]				
				NCTC 8325	ATCC 29213	ATCC 33591	ATCC 43300	MRS 394-1
None								
PW_{11}[d]	100μM	-	-	+1	+1	+7	+7	+8
SiW_{11}[d]	100μM	-	-	+1	+1	+8	+8	+8
p-Chloroaniline	5mM	*micF* *osmY*	337 289	+1	+1	+3	+7	+4
N, N-Dimethylformamide	10%	*clpB* *micF*	253 279	+1	0	0	+1	0
Dimethylsulfoxide	10%	*micF*	267	0	0	0	0	0
Doxorubicin	25μM	*clpB* *dinB* *recA* *umuDC*	254 265 264 286	+1	+1	+2	+5	+1
Ethanol	10%	*clpB* *merR* *micF*	384 288 325	+1	0	0	0	0
$HgCl_2$	1μM	*merR*	>1000	0	0	0	0	0
H_2O_2	2mM	*dinD* *katG* *recA* *umuDC*	270 294 356 295	0	0	0	0	+1
Methotrexate	25μM	*dinB* *recA* *umuDC*	267 327 290	0	0	0	0	0
Mitomycin C	200nM	*dinB* *dinD* *recA* *umuDC*	265 286 351 292	+1	+1	+3	+4	+5
NaCl[e]	+300mM	*osmY*	255	0	0	0	0	0
$NaNO_2$	100mM	*micF* *osmY*	264 331	+1	0	0	+1	+1
Paraquat	50μM	*micF* *nfo* *soi28* *zwf*	265 336 388 357	0	0	0	0	0
1-Propanol	10%	*clpB* *micF*	271 293	+1	0	0	0	0
Sucrose	200mM	*osmY*	254	0	0	0	0	0

In order to clarify the mechanism of action of POTs, reporter gene assay was performed using special bacterial strains (*E.coli*) transformed with a galactosidase gene-fused stress promoter [95]. The experimental details and references of every promoter gene are described in the author's report [50].

[a]When the induction rate was less than 250%, the data were omitted in order to minimize this table.

[b]The effect of various compounds on the susceptibility to oxacillin (MPIPC) is expressed as 'Enhancement Index' = $\log_2$ ((MIC of MPIPC without agent)/(MIC of MPIPC with agent)) [42, 43, 48, 49]. For instance, "+7" means that a compound made the strain (2^7=)128-fold susceptible to MPIPC. The established bacterial strains used in the experiments are described in our reports (NCTC8325/ATCC29213 = MSSA, ATCC33591/ATCC43300/MRS394-1 = MRSA) [42-49].

[c]Means of two independent triplicate determinations (n = 6) are shown here.

[d]500μ M PW_{11}/SiW_{11} induced 156/172% *osmY* and 127/160% *clpB*, respectively (others were unremarkable) [50].

[e]The culture medium contained salt to some extent, and the additional amount of NaCl is shown here.

In an aqueous solution, POTs (especially in low concentrations such as below mM order) may be partially decomposed and/or converted into other molecular species with or without binding some other substances present in the medium. Therefore, the "real" substances effective to MRSA are unknown in the strict sense of chemistry, although the starting compounds (*e.g.*, PW_{11}, SiW_{11}) are pure. ^{183}W NMR study is sometimes useful to solve such an issue, but this is quite insensitive when the concentration is very low. However, results of an elementary analysis

suggest that POTs incorporated into the membrane fraction of an MRSA strain seem to be intact [47]. It seems likely that neither molecular degradation nor decomposition of POTs is involved in the process sensitizing MRSA.

MECHANISMS OF THE ACTION OF POTS

Although the mechanism of sensitizing action of POTs has not been completely elucidated, POTs seem to reduce the expression of PBPs, thereby making MRSA susceptible to β-lactams. In addition, the effect of POTs is due to neither direct inactivation of PBP2' nor an increase in its affinity for β-lactams [43, 44, 46, 47, 49]. With this phenomenon, the following hypotheses are considered:

(A). Is the Sensitizing Effect Related to Oxidative Stress?

Interestingly, some POTs are highly accumulated in the membrane fraction of the bacterial cell and can be reduced *in vivo* [47]. It is well known that the oxidation state of some tungsten atoms in a POT molecule is decreased from W(VI) to W(V) when the compound is reduced [27-30]. Therefore, it is possible that such a reduced POT produces active oxygen species (*e.g.*, superoxide anion (O_2^-)) by autooxidation of W(V). However, it was recently found that tungsten compounds do not directly produce active oxygen species (see also the "Appendices" section). Their ability to cause oxidative stress seems to be weak, if even present at all [50]. In addition, H_2O_2 and paraquat, which were used as positive controls to produce an oxidative stress, did not sensitize MRSA strains to β-lactams (Table **1**). Since *S.aureus* is an aerobic and Gram-positive bacterium [15], the cells are most likely protected by a certain antioxidant system to overcome weak oxidative stress.

(B). Is the Sensitizing Effect Related to Polyanionic Characteristics?

It is now considered that the high negative charge density of the POT molecule is closely related to the sensitizing effect, because the effect on MRSA is diminished by "neutralizing" POTs with polycations such as protamine [42, 44, 48]. As described in the "Appendices" section, this is probably because these POTs will act as nucleic acid-analogues and inhibit enzymes with nucleic acids as their substrates (Fig. **4a**) [50].

Among compounds tested, mitomycin C (MMC) and doxorubicin (DXR) were found to have a sensitizing effect on MRSA strains similar to POTs (Table **1**). Since these two compounds are both inhibitors of DNA/RNA synthesis [51], this result seems to be compatible with the hypothesis that POTs sensitize strains of MRSA by inhibiting enzymes with nucleic acids as their substrates.

However, the selective toxicity (=MIC/effective concentration maximizing synergism to β-lactams) of MMC (600nM/200nM = 3) and DXR (150μM/25μM = 6) was much inferior than that of POTs (*e.g.*, 2000μM/50μM = 40 [48]). The reason is unclear, but there may be a unique mechanism specific for POTs to sensitize MRSA strains to β-lactams.

(C). Why is the Resistance to β-Lactams of MRSA Strongly Reduced by POTs?

It is well accepted that the resistance of MRSA to β-lactams is principally attributed to the acquisition of PBP2' having lowered affinity to β-lactams [17, 18, 20-23]. Although POTs reduced the expression of ordinary PBPs (PBP1~4), the suppressive effect was stronger against PBP2' [43, 47, 49]. As PBP2' is a fragile protein with a possibly rapid turnover [25] and relatively poor enzymatic activity [52], MRSA strains need to constantly produce enough PBP2' for full expression of β-lactam resistance. Thus, this resistance will appear to be more easily affected than other phenotypic characteristics, even though several (anion-sensitive) ordinary cell functions such as transcription and translation activity were only weakly inhibited by POTs (Fig. **4a**). Otherwise, there may be some unique regulation system specific to PBP2'.

(D). Is there Any Other Possibility of the Sensitizing Effect?

Among substances tested, it was found that *p*-chloroaniline (pCA) also produced a sensitizing effect on MRSA strains (Table **1**), and this is a positive control of the *osmY* promoter gene (known to be induced by osmotic stress [53]). However, a relatively high concentration (5mM) was required for pCA to sensitize the MRSA strains (*i.e.*, 1mM pCA did not show any observable effect on MRSA strains, but PW_{11}/SiW_{11} was still active even at 10~25μM). In addition, the selective toxicity of pCA (15mM/5mM = 3) was much less than that of PW_{11}/SiW_{11} (2000μM/50μM = 40). Likely, the action mechanism of pCA may be somewhat different from that of POTs.

It is generally accepted that many chemical and/or physical factors can induce some stress response from a living cell, including bacteria. Interestingly, POTs seemed to induce an osmotic stress on the bacterial cells of *Escherichia coli* [50], which is a common feature of pCA and POTs. Both have a sensitizing effect on MRSA and can induce an osmotic signal in *E.coli* [50]. If these two phenomena are closely related to each other, one can consider the idea that the sensitization of MRSA to β-lactams by POTs is a type of stress response. POTs may cause a growth-arrest signal on the bacterial cells, which will cause decreased expression of PBPs. This matter will be discussed further in the next section.

(E). What does an Osmotic Signal Mean?

Interestingly, it was found that some POTs induced the *osmY* gene expression in *E.coli* (Table **1**) [50]. Usually, this gene is induced by an osmotic signal [53], but the effect of POTs occurred at a much lower concentration than other ordinary salt (*e.g.*, NaCl induced *osmY* only ~2 fold at 200mM) (Table **1**).

Since the constituents of POTs (*e.g.*, HPO_4^{2-}, WO_4^{2-}) showed almost no effect on this promoter gene [50], a chemical feature unique to POTs and originating from neither of their constituents (*i.e.*, a polyanionic characteristic) may play an important role in the above result. In addition, cationic peptide antibiotics such as

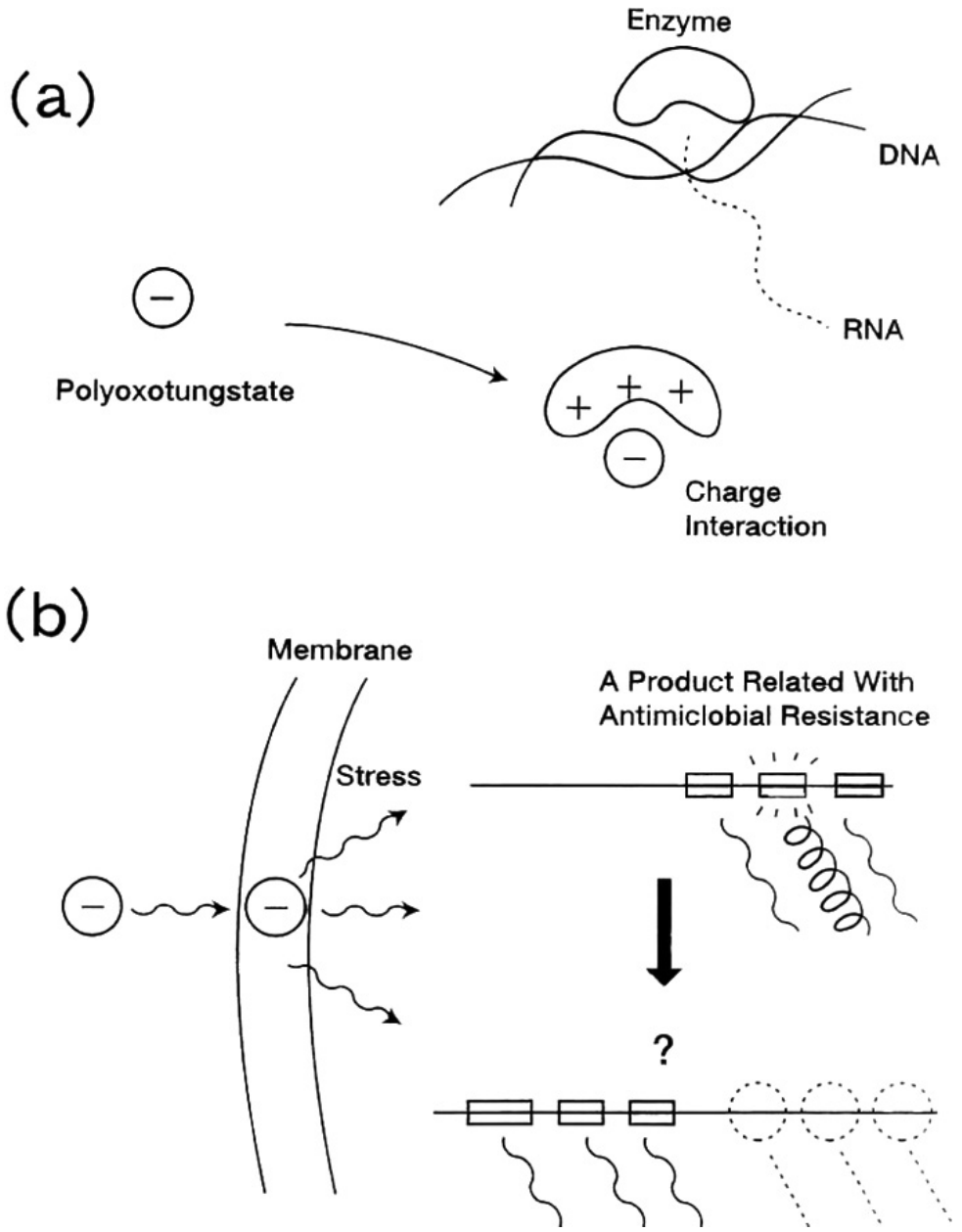

Fig. (4). Possible mechanism of action of POTs on the sensitizing effect on MRSA.

The author's current hypothesis is schematized.

(a) POTs are a type of polyanion, and they will commonly and universally act as nucleic acid-analogues. It is possible that POTs inhibit such anion-sensitive enzymes with nucleic acids as their substrates nonspecifically as a result of charge interaction.

(b) It is generally accepted that many chemical and/or physical factors can cause some stress response from living cells, including bacteria. If we can change the intrinsic conditions of bacterial cells having some antimicrobial resistance by interacting with some stress-producing compound like POTs, we may easily overcome the drug-resistance of many pathogens (*i.e.*, we need not directly inactivate a gene product causing drug-resistance, which is sometimes insensitive to many antimicrobials).

polymyxin B were recently shown to selectively induce *osmY* [54]. These findings suggest that the osmotic sensor protein related to this promoter gene is sensitive to such a charged substance. Although the detailed mechanism of *osmY* gene expression has not yet been elucidated, it is possible that a type of charge interaction may be involved in this system.

(F). Is there Any Relationship between the *OsmY* Gene and Drug Resistance?

It is known that the expression of *osmY* in *E.coli* is regulated by an alternate sigma cofactor (σ^s) of RNA-polymerase, which is translated from the *rpoS* gene in the stationary phase of the cell cycle [53]. Therefore, it is possible that the *osmY* gene expression induced by POTs reflects a simple alteration of growth conditions rather than

osmotic stress. However, POTs did not strongly suppress the bacterial growth (measured by optical density of the culture medium) [49, 50].

Although the relationship between the sensitizing effects on MRSA and the expression of *osmY* in *E.coli* is yet to be elucidated, it is possible that the POT-induced sensitization of MRSA to β-lactams may be related to some alterations and/or modifications in a certain signal transduction system as noted elsewhere [55]. In this line of thought, several exogenous factors can change various phenotypes of a bacterial cell (which may include factors related to virulence and/or drug resistance) by interfering in signal transduction.

It is well known that the *rpoS* gene product (σ^s) functions as a regulator of several genes in *E.coli* [52]. Under the conditions where σ^s is induced, some (*rpoS*-dependent) genes will be selectively expressed, while the expression of other genes may be decreased instead (Fig. **4b**). Interestingly, it seems likely that some PBPs of *E.coli* are also regulated by the σ^s [56]. This paper will further explore the possibility that POTs induce a change of intracellular conditions, resulting in a reduced drug resistance.

It is supposed that a situation in which the alternative sigma cofactor (like the σ^s in *E.coli*) is expressed would possibly be hazardous to the bacterial cell, so that the cells may stop growth by decreasing activities of some functional units related to cellular proliferation (which may include PBPs and/or their regulators). This idea may provide a hint for the development of a new antimicrobial agent (Fig. **4b**).

SUBSTANCES/FACTORS REDUCING β-LACTAM-RESISTANCE OF MRSA

In addition to POTs, it was reported that several substances (*e.g.*, Triton X-100) also sensitize MRSA strains to β-lactams (listed below). Although their sensitizing mechanisms are unknown, some of their effects seem to be unrelated to PBP2'. Therefore, we can expect a strong synergistic effect when these agents with different sites of action are combined effectively. One hopes that such a synergy will drop a hint to promote a new strategy of antimicrobial chemotherapy in the near future.

(1). Acid and Heat

It is known that strains of MRSA become susceptible to β-lactams when they are incubated under acidic conditions (*e.g.*, pH 5) or at a relatively high temperature (*e.g.*, 42°C). This is due to the thermal- and pH-fragility of PBP2' [25], and thus, hyperthermic treatment may be possible. Although POTs are slightly acidic compounds, the sensitization by POTs are not an acidic effect, since the pH of the culture medium was kept around pH 7 throughout the experiments [42-50].

(2). TOC-39

TOC-39 is a unique cephem antibiotic with strong affinity to PBP2' [57, 58], thereby killing strains of MRSA all alone. Therefore, the synergism of TOC-39 with POTs appears to be weak, since this cephem had previously acted on MRSA prior to being sensitized by POTs [59].

(3). Fosfomycin

It was reported that fosfomycin (FOM) has a similar sensitizing effect to POTs and MRSA becomes susceptible to β-lactams when cultured with FOM [60]. This effect is considered to be the result of the phosphate radical present in the FOM molecule, which inhibits the production of a murein monomer at an early stage by competing with phosphoenolpyruvate. Then, the amount of inducible PBPs (such as PBP1, PBP2 and PBP4, including PBP2'), except for one constitutive form (PBP3), is reduced markedly, since expression and/or transcription from the PBP-encoding gene will be suppressed when the material of the cell wall is depleted [60, 61].

However, the sensitizing effect of POTs is still evident even in highly FOM-resistant strains [42, 49], and the effect is not related to FOM-susceptibility. It is known that the concentration of FOM required to produce the sensitizing effect is generally more than 1/4 of the MIC [60, 61], at which the bacterial growth is retarded to some extent, but POTs are effective below 1/40 of the MIC [43, 48, 49]. In addition, synergism between FOM and β-lactams did not always occur against all the strains of *S.aureus* [61], yet there is no strain against which POTs are ineffective.

In the literature, it is reported that other classes of antimicrobials also have a synergism to β-lactams (generally more than 1/4 of the MIC is required), but their mechanism of synergism and their relationship to POTs are unclear [62].

(4). Detergents and Triazine Dye

Interestingly, several detergents (*e.g.*, Triton X-100 [63]) and a triazine dye (*e.g.*, cibacron blue F3GA [64]) sensitize strains of MRSA to β-lactams. They seem to have a common feature in that they are lipophilic compounds. Since POTs effective to MRSA strains are highly accumulated in the membrane fraction [47], although they are ionic and highly water-soluble compounds [27-30], there may be a common mechanism of action among POTs and these compounds. However, it was reported that the sensitizing effects of these detergents and triazine dye were not related to PBP2' [65, 66].

(5). Catechins

Several catechins (*e.g.*, epigallocatechin [67, 68], tannin [69]) are reported to sensitize strains of MRSA to β-lactams. Curiously, similar to POTs [42-49], there seems to be no synergism of catechins with other classes of antibiotics [70]. Although the detailed mechanism of action is unclear, it seems likely that catechins directly interact with peptidoglycan in the cell wall and disturb its integrity [71].

(6). The others

In the literature, it is reported that farnesol [72] and several flavonoids [73, 74] have an antibacterial effect on MRSA and a synergism with β-lactams, but their mechanism of action is quite unknown.

APPENDICES

I. The Basis of the Chemistry of Tungsten

Tungsten (W) is an important material in industry where it is used to make electrodes and filaments of photolamps. In addition, this metal is also a component of several hard alloys [75-77]. The most stable oxidation state of tungsten is W(VI), but only oxoanionic molecular species (such as WO_4^{2-}) are stable under aqueous conditions [75, 76]. This is probably because the large positive charge of the atomic nucleus pulls the electron shell strongly towards the nucleus. Therefore, even if W^{6+} does exist in aqueous solution, it will pull out negatively charged oxygen atoms from water molecules (H_2O) with the formation of WO_4^{2-} as follows (unless the activity of the solution is extremely high): $W^{6+} + 4H_2O \rightarrow WO_4^{2-} + 8H^+$.

The most familiar tungsten compound is sodium tungstate (Na_2WO_4) and its derivatives. Interestingly, WO_4^{2-} has the unique feature that this oxoanion polymerizes easily with itself or with other oxoanions such as PO_4^{3-}, forming a POT [75, 27-30]. For instance, heating a mixture of WO_4^{2-} and PO_4^{3-} under acidic conditions causes the formation of dodecatungstophosphate ($[PW_{12}O_{40}]^{3-}$, PW_{12}) as follows (unless the acidity of the solution is extremely high): $12WO_4^{2-} + PO_4^{3-} + 24H^+ \rightarrow [PW_{12}O_{40}]^{3-} + 12H_2O$.

PW_{12} is partially hydrolyzed at around neutral pH as follows: $[PW_{12}O_{40}]^{3-} + 6OH^- \rightarrow [PW_{11}O_{39}]^{7-} + WO_4^{2-} + 3H_2O$, and the major polyanion present in the solution is PW_{11} [27-30]. Although PW_{11} is decomposed completely at pH > 8, 80~90% of this compound still remains as $[PW_{11}O_{39}]^{7-}$ below pH 7.2 [78, 79]. It therefore seems natural to investigate the biological and biochemical effects of WO_4^{2-} and PW_{11}, both of which are representative of the tungsten compounds present around physiological (neutral) pH.

II. Classical Uses of Tungsten Compounds

In addition to the example shown in Fig. **1**, many other species of POTs are known to date [27-30]. However, the biological and biochemical effects (including the toxicity) of tungsten compounds are unclear, despite their importance in industrial processes (*e.g.*, tungsten compounds are reported to be useful as a catalyst in organic synthesis) [28]. Tungsten compounds are used only to a limited extent in biochemistry and medical chemistry as follows:

(i). Tungsten compounds are used as a staining agent in pathology, particularly in electron microscopy (known as "negative stain") [80]. PW_{12} is usually used for this purpose as this compound is highly opaque to X-rays. PW_{12} binds well to certain cellular and/or subcellular components (*e.g.*, carbohydrates, mucopolysaccharides), but this feature seems to be pH-dependent. Uranium compounds (such as UO_4^{2-}) are also frequently used for this purpose as the resolution power of PW_{12} staining is relatively low, although PW_{12} generally gives higher contrast. In addition, PW_{12} is sometimes used in sample preparation in light microscopic studies, a technique known as "Mallory's phosphotungstic acid-hematoxylin staining" [81].

(ii). Tungsten compounds are also used as a precipitating agent for low-density lipoproteins (LDLs) [82, 83]. Usually, PW_{12} and magnesium (Mg^{2+}) are used for this purpose (*e.g.*, the measurement of cholesterol in high-density lipoproteins after separation from LDLs). LDLs bind negatively charged materials such as dextran sulfate, and Mg^{2+} helps PW_{12}-bound LDLs precipitate. In addition to LDLs, PW_{12} are known to interact with various materials, probably because of a charge interaction [84, 85]. For instance, PW_{12} is sometimes used as a potent precipitating agent for proteins and alkaloids [86, 87].

(iii). Tungsten compounds, particularly PW_{12}, are used as colorimetric agents for reductants such as ascorbate [88]. PW_{12} solution is unique in that it turns intense blue-purple when adequately reduced. This is because some tungsten atoms in the PW_{12} molecule are reduced from W(VI) to W(V). The colored substance thus formed is called "tungsten blue" or "heteropolyblue", as POT, including heteroatoms in molecules such as PW_{12}, is sometimes known as "heteropolyacid" [27-30]. Although this reaction is nonspecific, it is frequently applied in the field of analytical chemistry [28]. The Lowry method for protein quantification is a well-known application of "tungsten blue" formation [89].

(iv). Molybdenum-paucity is induced by chronic administration of WO_4^{2-} (*e.g.*, the activity of xanthine oxidase, a molybdenum enzyme, is decreased). This seems to be a simple substitution effect because the chemical properties of tungsten are very similar to those of molybdenum [75-77]. Although some prokaryotes are known to require tungsten as a cofactor of several enzymes, it is not thought that tungsten is essential for eukaryotes [90]. Some investigators have reported that WO_4^{2-} and molybdate (MoO_4^{2-}) stimulated the adenylate cyclase activity in rat brains [91, 92], but the physiological significance of this is unclear.

III. RECENT ADVANCES IN THE BIOLOGY AND BIOCHEMISTRY OF TUNGSTEN COMPOUNDS

Advances in biological and biochemical techniques have allowed the demonstration of some unique properties of tungsten compounds:

(1). It is generally accepted that WO_4^{2-} has relatively low toxicity compared with compounds of other heavy metals such as mercury (Hg). This is probably due to its oxoanionic molecular form, which is highly water-soluble [76, 77]. This compound is excreted rapidly into urine and is not believed to accumulate in the body, except for organs such as bone and the spleen, even after chronic exposure. There have been only a few case reports of acute intoxication due to tungsten compounds [93]. In addition, the direct toxicity of PW_{11}/PW_{12} to cultured human cells is low and its growth inhibitory concentration is >100μ M [94].

There is no evidence to suggest that tungsten compounds are carcinogenic or mutagenic [76, 77]. This is supported by the findings that neither WO_4^{2-}

nor PW_{11}/SiW_{11} induces DNA damage-sensitive promoter gene expression [50], namely, *ada*, *dinB*, *dinD*, *nfo*, *recA* and *umuDC* [95]. Furthermore, neither WO_4^{2-} nor PW_{11}/SiW_{11} induces *merR* gene expression [50], whereas other heavy metal compounds such as $HgCl_2$ do it very strongly [95]. This suggests that the mechanisms of action of these tungsten compounds *in vivo* are distinct from those of other (cationic) heavy metals.

(2). It has been reported that chronic exposure to dust containing tungsten metal can induce pulmonary fibrosis. However, this does not seem to be due to tungsten itself, but to the toxic effect of active oxygen species generated by interaction with cobalt (Co) [96].

In our recent study, neither WO_4^{2-} nor PW_{11}/SiW_{11} were found to induce *soi28* and *katG* gene expression [50], both of which are known to be induced by compounds such as paraquat and hydrogen peroxide (H_2O_2), which cause oxidative stress [95]. The *zwf* gene is also known to be induced by oxidative stress [95], but neither WO_4^{2-} nor PW_{11}/SiW_{11} were found to induce this gene [50]. Furthermore, the amount of H_2O_2 in cell culture medium did not increase significantly by the addition of these tungsten compounds (measured by chemiluminescence). These findings suggest that the biological and biochemical effects of WO_4^{2-} and PW_{11}/SiW_{11} are not related to active oxygen species.

(3). Some investigators have reported that tungstate gel induces generalized seizures when injected intracranially, but the mechanisms responsible for this are unclear [97-99]. As the tungstate gel was prepared by mixing Na_2WO_4 solution with a strong acid, it would have been a mixture of several POTs such as metatungstate ($[W_{12}O_{39}]^{6-}$) [27, 30, 75]. Studies in our laboratory showed that PW_{11} induced seizures, but Na_2WO_4 alone did not (details to be published elsewhere). The epileptogenic character of PW_{11} and tungstate gel may be related to their polyanionic character and probably not to the character of tungsten itself as a heavy metal.

PW_{11} has been found to have anion channel-blocking activity in human erythrocytes and porcine sperm [32, 38, 100], probably because of its high negative charge (*i.e.*, PW_{11} would occupy the active center of the anion transporter). If PW_{11} also inhibited the γ-aminobutyric acid (GABA)-gated chloride channel in the central nervous system [101], as well as that of erythrocytes, the epileptogenicity of this compound could be explained by the reduction of sedative activities mediated by the GABA-gated channel [102, 103]. This is possible because chloride channels in various cells are known to be structurally conserved [101].

(4). Many investigators have reported that WO_4^{2-}, like vanadium compounds [104], induces hypoglycemia. Although the mechanism for this is unclear, it is considered to be due to WO_4^{2-} mimicking the action of insulin [105], or to the restoration of islet function [106]. However, some investigators have explained this phenomenon as a type of stress reaction [107], while others reported that WO_4^{2-} potentiated insulin secretion [108] and also inhibited glucose-6-phosphatase activity [109]. Recent investigation demonstrated that WO_4^{2-} improves glucose metabolism in the liver [110]. Since there are many possible hypotheses, further research is clearly required to clarify this issue.

(5). PW_{11}/SiW_{11} have previously been shown to prolong blood coagulation time by activating antithrombin III (ATIII) in plasma, whereas their constituents (*e.g.*, WO_4^{2-}, HPO_4^{2-}) did not [38]. Since ATIII is universally activated by polyanionic substances such as heparin [111], this can be explained by the polyanionic character of PW_{11}/SiW_{11}, which seems to mimic that of heparin [27-30, 38, 39, 112].

Recently, serum triglyceride levels were found to decrease when PW_{11} was injected directly into the animal [113]. This may be due to an increase in plasma lipoprotein lipase (LPL) activity. Since LPL is known to be released from the endotherial cell surface into plasma by polyanionic substances [114], this observation can also be explained by the polyanionic character of PW_{11}. In addition, there is some evidence that tungsten compounds bind to certain proteins and alter their functions and/or properties [115-117]. Therefore, PW_{11} does appear to have an important biochemical effect even though it is only a simple, inorganic compound (see text, "The discovery of the biological effects of POTs" section).

(6). PW_{11} has recently been found to induce platelet aggregation *in vivo* and *in vitro*, whereas its constituents (WO_4^{2-} and HPO_4^{2-}) had no effect [113]. As PW_{11} is a heparin-like polyanion, this phenomenon can be considered to mimic heparin-induced thrombocytopenia (HIT) [118, 119]. When compared with the disease in humans, PW_{11}-induced thrombocytopenia occurred immediately and with higher reproducibility, meeting the criteria of "type I HIT" (*i.e.*, occurring in a non-idiosyncratic manner without participation of platelet-associated antibodies). Rather, in terms of severity, the thrombocytopenia meets the criteria of "type II HIT" [120].

A tungsten compound with potent antiviral activity ($[NaSb_9W_{21}O_{86}]^{18-}$, HPA-23) has been reported to cause thrombocytopenia in a dose-dependent manner, with abnormal blood coagulation time in humans [121]. The mechanism responsible for this adverse effect may be similar to that of PW_{11}. These tungsten compounds induce platelet aggregation *in vivo* probably due to a charge interaction with components on the platelet surface [113].

(7). It is known that many types of POTs inhibit the activity of enzymes with nucleic acids as their substrates (*e.g.*, restriction enzyme, polymerase), while POTs have almost no effect on many other ordinary enzymes [50, 122-124]. The mode of inhibition is generally "competitive" [125], meaning

that the phenomenon of enzyme inhibition is probably due to the polyanionic characteristics of POTs, which act as a nucleic acid-analogue as noted elsewhere [41, 125]. Because nucleic acids can be regarded as a type of polyanion, it is possible that POTs inhibit such anion-sensitive enzymes nonspecifically as a result of charge interaction (Fig. **4a**). This feature may be useful for long-term preservation of nucleic acids, in that POTs will protect DNA/RNA molecules from decomposition by trace contamination with nucleases if they are mixed with the solution.

In addition, it is generally accepted that many POTs such as SiW_{11} have a potent antiviral activity. This is considered to be due to the inhibition of RNA-dependent DNA polymerase [122-124]. Another possible mechanism of action responsible for the antiviral effect of POTs has recently been reported, namely, that such compounds can inhibit the fusion of viral particles to the host cell membrane [126].

As acid polysaccharides such as dextran sulfate are known to have a similar inhibitory effect on viral invasion into the host cell [127, 128], this antiviral activity can be considered to be molecular mimicry of POTs to polysaccharides. This is further supported by experimental evidence indicating that PW_{11}/SiW_{11} cause a metachromatic reaction [38], as is the case for acid polysaccharides such as heparin [129]. In any case, we think that the effects of POTs are principally based on their polyanionic characteristics.

(8). POTs can be used as phase-transfer agents for certain cationic materials. These compounds form an ion-pair with cationic materials, and the complex becomes extractable with organic solvents [130, 131]. Some investigators have applied this feature to potentiometric measurement [132-134], and a lacunary-substituted species of PW_{11} has been used as a charge standard in a colloid titration [135]. In addition, PW_{11} is useful for checking how deeply the charge interaction concerns a certain binding system [41]. These findings support the hypothesis that the mechanism behind the various effects of POTs is mainly charge interaction. Since POTs such as PW_{11}/ SiW_{11} are pure and single compounds, they can be used as standard substances for other polyanionic materials such as heparin [38, 135].

(9). POTs will interfere with certain binding systems where charge interaction plays an important role [41]. For instance, these compounds may be useful as eluents for affinity chromatography [39, 40]. It is reported that SiW_{11}/SiW_{12} efficiently solubilizes some components from subcellular organelles [136].

(10). As described in the text, POTs sensitize MRSA by decreasing the expression of PBP2' [42-49]. Recently, it was found that strains of MRSA with an extremely high resistance to β-lactams are easily detected by adding PW_{11}/SiW_{11} to the growth medium. When a POT is mixed with the culture medium, the growth inhibition zone formed around a paper disk containing a β-lactam antibiotic was significantly enlarged (Fig. **3**) [45, 48, 49]. However, colonies did appear inside the inhibition zone when the MRSA strain was highly resistant to the antibiotic. It is noteworthy that the geometric pattern of the colonies was roughly related to the result of the population analysis [137]. When POTs and β-lactams are used in an appropriate combination, we may estimate the mechanism of resistance working in an MRSA strain, which could possibly lead to the use of POTs as a "tool" in laboratory tests in clinical microbiology, as suggested elsewhere [59].

For this purpose, SiW_{11} (or its derivatives) may be more suitable than PW_{11} because SiW_{11} has better pH stability. Around 10% of SiW_{11} is decomposed with every 0.1 increase in pH from pH 8.0 (completely hydrolyzed over pH 9), although PW_{11} starts to decompose from pH 7.0 [27].

ABBREVIATIONS

AC	=	Adenylate cyclase
ATIII	=	Antithrombin III
DIDS	=	4, 4'-diisothiocyanatostilbene-2, 2'-disulfonate
DMPPC	=	Dimethoxyphenylpenicillin (methicillin)
DXR	=	Doxorubicin
FOM	=	Fosfomycin
GABA	=	γ-aminobutyric acid
HIT	=	Heparin-induced thrombocytopenia
LDL(s)	=	Low-density lipoprotein(s)
LPL	=	Lipoprotein lipase
MIC(s)	=	Minimum inhibitory concentration(s)
MMC	=	Mitomycin C
MPIPC	=	Methylphenylisoxazolylpenicillin (oxacillin)
MRSA	=	Methicillin-resistant *Staphylococcus aureus*
MSSA	=	Methicillin-susceptible *S.aureus*
PBP(s)	=	Penicillin-binding protein(s)
pCA	=	*p*-Chloroaniline
PCase	=	Penicillinase
PCG	=	Penicillin G
PDE	=	Phosphodiesterase
PK	=	Protein kinase
POT(s)	=	Polyoxotungstate(s)
PW_{11}	=	Undecatungstophosphate ($[PW_{11}O_{39}]^{7-}$)
PW_{12}	=	Dodecatungstophosphate ($[PW_{12}O_{40}]^{3-}$)
SiW_{11}	=	Undecatungstosilicate ($[SiW_{11}O_{39}]^{8-}$)
σ^s	=	Alternative sigma cofactor

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