

# Antibacterial Evaluation of Novel Organoarsenic Compounds by the Microcalorimetric Method

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**Abstract** Antibacterial activities of novel organoarsenic compounds As(III)-containing Schiff bases on *Escherichia coli* (CCTCCAB91112) were investigated by microcalorimetry in this study. The experimental result showed that the arsenic(III)-containing Schiff bases at micromolar concentration exhibit strong inhibition on the *E. coli*. Specifically, the growth rate constant  $k$  decreased, and the generation time  $t_G$  and the inhibitory ratio  $I$  (percentage) increased with the increased dose of the arsenicals as inhibitors. All of the arsenicals display the feature of considerable lag phase inhibition on the cell growth. The compound 4-(4-bromobenzaliminyl)phenylarsenoxide makes the lag phase of *E. coli* cell growth cycles to reach 650 min at 5  $\mu\text{mol/L}$ . The compounds with donating electron groups at aromatic ring B have lower  $\text{IC}_{50}$  to present higher antibacterial activity. The compound 4-(4-hydroxyl-3-methoxylbenzaliminyl)phenylarsenoxide has the lowest  $\text{IC}_{50}$  (1.82  $\mu\text{mol/L}$ ) to show the strongest antibacterial activity among them.

**Keywords** Organoarsenic compounds · *Escherichia coli* · Microcalorimetry · Antibacterial activity

## Introduction

Although many infectious diseases can now be controlled, microorganisms can still be a major threat to human health.

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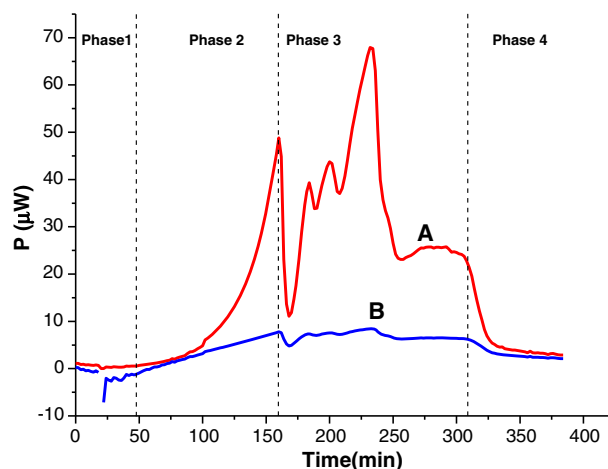
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Particularly in developing countries, microbial diseases are still major causes of death, and millions still die yearly from such microbial diseases as malaria, tuberculosis, cholera, African sleeping sickness, measles, pneumonia, and diarrheal syndromes. In addition, human beings worldwide are under a threat from diseases that could emerge suddenly, such as the Severe Acute Respiratory Syndrome outbreak in 2002 in China, bird flu, and Ebola hemorrhagic fever, which are primarily animal diseases that, under certain circumstances, can be transmitted to human and spread quickly through a population [1–4]. Another current example is a well-understood microorganism *Escherichia coli* that still brought on a big trouble to Europe because of severe foodborne disease due to enterohaemorrhagic *E. coli* [5]. Therefore, there is a need for more and effective antibacterial agents at hand to deal with ordinary or sudden health threat from microorganisms.

Arsenicals as effective antibacterial drugs were used in the world more than 2,000 years ago to treat a variety of diseases including syphilis and cancer [6]. In the eighteenth century, Thomas Fowler developed an oral inorganic arsenical-based therapeutic agent known as “Fowler's solution” to treat a number of malignant diseases including leukemia, Hogkin's disease, pernicious anemia, and so on. Specifically, the first synthetic organoarsenical used for medical treatment purpose, salvarsan, was developed about 100 years ago by Paul Ehrlich as the most effective prescribed drug to treat African trypanosomiasis and syphilis [7, 8]. The arsenical-based treatments were abandoned due to concerns about its toxicity after penicillin and other antibiotics became available at the mid-twentieth century. But later discovered ability of arsenic trioxide to treat successfully acute promyelocytic leukaemia has radically changed its fate of being abandoned [9–12]. This inspiring discovery has reignited the enthusiasm of medicinal chemists for arsenical-based drugs.

Arsenic is the 33rd element with higher toxicity as well as higher bioactivity in the Periodic Table. Arsenic has two biologically important oxidation states, As(III) and As(V). As(III) species, in particular, exerts a wide array of effects in biological systems mainly because of reactivity of As(III) as a soft metal, reacting selectively with vicinal thiols in biological macromolecules to form a strong As-S bond [13, 14]. There are, therefore, many potential intracellular and extracellular targets for arsenic attack such as some thiol-containing enzymes. Such enzymes include dehydrogenases (e.g.,  $\beta$ -hydroxybutyrate dehydrogenase, pyruvate dehydrogenase, lipoic acid dehydrogenase), oxidases (e.g., cytochrome oxidase), and urease [15, 16]. Arsenic binding also impacts proteins involved in signaling pathways [17] and thiol proteins within the mitochondrion [18]. As(III) is much more toxic than As(V), and inorganic arsenical is more toxic than organoarsenical in general [19]. Organoarsenicals have better affinity on the biological macromolecules, subcellular organelles, and cells.

In an effort aiming at evaluating the antibacterial activity of organoarsenicals with a more favorable toxicity profile, we synthesized a new class of organic arsenicals (Table 1). They are a type of arsenic (III)-containing Schiff bases derivatives, i.e., small organoarsenical molecules with two bioactive groups, arsenic(III) group and imine group. Schiff bases have been shown to exhibit a broad range of bioactivities, including

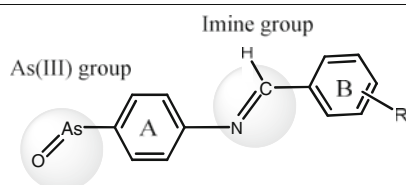


**Fig. 1** a The metabolic power–time curve recorded from *E. coli* transformants growing in LB medium, at 37.0 °C, 0  $\mu$ M drug, b natural logarithm of A

antibacterial, antiproliferative, and antiviral activities [20, 21]. In these Schiff bases, the imine group is critical to their biological activities. In our work, the activities of arsenic (III)-containing Schiff base derivatives against microorganism were evaluated by using *E. coli* (CCTCCAB91112) as the testing bacterial strain via the microcalorimetric method. Microcalorimetry is one of the most important techniques and methods

**Table 1** The chemical structure and name of the arsenic (III)-containing Schiff base derivatives with dual functional groups: As(III) and imine group, two aromatic rings: A and B

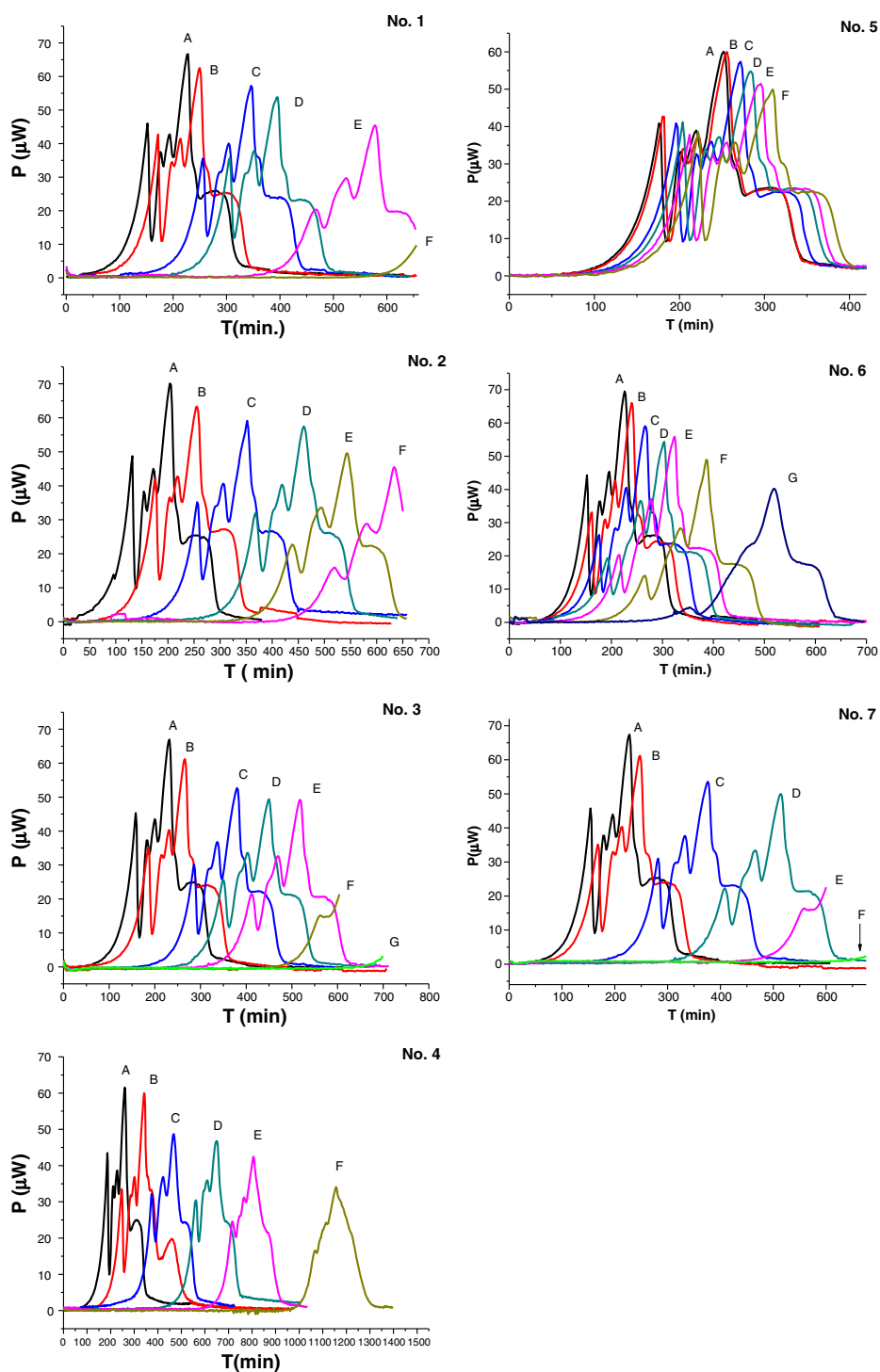
No.	R	Name
1	2-hydroxyl	4-(salicyliminyl)phenyl arsenoxide
2	4-hydroxyl-3-methoxyl	4-(4-hydroxyl-3-methoxybenzaliminyl)phenyl arsenoxide
3	2,4-di hydroxyl	4-(2,4-dihydroxybenzaliminyl)phenyl arsenoxide
4	4-bromo	4-(4-bromobenzaliminyl)phenyl arsenoxide
5	4-aza	4-(4-azabenzaliminyl)phenyl arsenoxide
6	—	4-(3-indolaliminyl)phenyl arsenoxide
7	—	4-(9-anthraliminyl)phenyl arsenoxide



currently for thermochemistry and thermodynamic studies [22]. The various metabolic events in living systems are actually biochemical reactions having an associated heat exchange between the system and its surrounding. By monitoring the thermal effect with a heat-sensitive calorimeter, microcalorimetric method can directly determine the biological activity of a living system and provide a continuous measurement of thermal production, thereby giving much information about

the metabolism of organisms in both qualitative and quantitative ways [23–25]. The microcalorimetric method has been widely applied in life sciences as well as environmental, material, medical, clinical, pharmaceutical sciences for its high sensitivity, detectability and accuracy, and its non-invasive, non-destructive analysis and its convenience in realizing automated, continual, real-time, and on-line analysis [26, 27].

**Fig. 2** The thermal spectrograms (metabolic power–time curves) of the *E. coli* growing in the culture media with and without addition of arsenicals No. 1–7 as inhibitors at different concentrations under controlling conditions (37.0 °C culture temperature, 5 mL of LB culture media). *No. 1*: 4-(salicyliminyl)phenyl arsenoxide; *A* control, *B* 2  $\mu\text{M}$ , *C* 4  $\mu\text{M}$ , *D* 6  $\mu\text{M}$ , *E* 8  $\mu\text{M}$ , *F* 108  $\mu\text{M}$ . *No. 2*: 4-(4-hydroxyl-3-methoxybenzaliminy)phenyl arsenoxide; *A* control, *B* 0.2  $\mu\text{M}$ , *C* 0.5  $\mu\text{M}$ , *D* 1.0  $\mu\text{M}$ , *E* 1.5  $\mu\text{M}$ , *F* 2.0  $\mu\text{M}$ . *No. 3*: 4-(2, 4-dihydroxybenzaliminy)phenyl arsenoxide; *A* control, *B* 1  $\mu\text{M}$ , *C* 1.5  $\mu\text{M}$ , *D* 2  $\mu\text{M}$ , *E* 3  $\mu\text{M}$ , *F* 4  $\mu\text{M}$ , *G* 5  $\mu\text{M}$ . *No. 4*: 4-(4-bromobenzaliminy)phenyl arsenoxide; *A* control, *B* 1  $\mu\text{M}$ , *C* 2  $\mu\text{M}$ , *D* 4  $\mu\text{M}$ , *E* 5  $\mu\text{M}$ , *F* 10  $\mu\text{M}$ . *No. 5*: 4-(4-azabenzaliminy)phenyl arsenoxide; *A* control, *B* 2  $\mu\text{M}$ , *C* 4  $\mu\text{M}$ , *D* 6  $\mu\text{M}$ , *E* 8  $\mu\text{M}$ , *F* 10  $\mu\text{M}$ . *No. 6*: 4-(3-indolaliminy)phenyl arsenoxide; *A* control; *B* 1  $\mu\text{M}$ , *C* 1.5  $\mu\text{M}$ , *D* 2  $\mu\text{M}$ , *E* 3  $\mu\text{M}$ , *F* 4  $\mu\text{M}$ , *G* 5  $\mu\text{M}$ . *No. 7*: 4-(9-anthraliminy)phenyl arsenoxide; *A* control, *B* 1  $\mu\text{M}$ , *C* 2  $\mu\text{M}$ , *D* 3  $\mu\text{M}$ , *E* 4  $\mu\text{M}$ , *F* 10  $\mu\text{M}$



## Materials and Methods

### Strain and Culture Medium

The bacterial strain, *E. coli* (CCTCCAB91112), was provided by the Chinese Center for Type Culture Collections, Wuhan University, Wuhan 430072, People's Republic of China. The *E. coli* was activated on a peptone-sterilized culture in isothermal bioreactor at 180 rpm of circumrotating frequency, 37 °C for 8 h. The activated *E. coli* was inoculated into Luria-Bertani (LB) liquid culture medium before testing. The peptone culture medium contained per 1,000 mL (pH=7.2): NaCl 5 g, peptone 10 g, and beef extract 6 g. The LB culture medium contained per 1,000 mL (pH=7.2): NaCl 5 g, peptone 10 g, and yeast extract 5 g. They must be sterilized at a high pressure of  $1.034 \times 10^5$  Pa steam at 120 °C for 30 min before inoculation.

### Equipment

The heat conduction microcalorimeter, LKB-2277 Bioactivity Monitor (Thermometric AB, Sweden), was used to obtain the thermogenic curves of drug/*E. coli* interaction. The equipment was thermostated at 37.0 °C. The operating mode for the monitoring was stopped-flow mode.

### Experimental Procedure and Method

The manipulation requires an aseptic technique, a series of steps to prevent contamination during manipulations. Sample tubes were quantitatively charged with 5 mL culture medium with an *E. coli* (CCTCCAB91112) inoculum. The sample cells of the calorimeter were cleaned and sterilized sequentially with 0.1 mol/L HCl, 0.1 mol/L NaOH, 75 % alcohol, and sterilized distilled water. After the system was run to a stable baseline, the sample in tubes was pumped into the sample cells via a LKB-2132 micropex peristaltic pump at a flow rate of 50 mL h<sup>-1</sup>. The monitor was opened, and heat production rate was recorded at intervals of 60 s. In every turn, at least two tunnels can be used simultaneously for monitoring two samples.

At the end of every turn, an integral thermal power–time function curve was given corresponding to a growth cycle of the *E. coli* population including four phase: lag phase, exponential phase, stationary phase, and death phase. The cell cycle of *E. coli* contains a period of indeterminate length that reflects a stochastic reaction, beginning at some time after a round of chromosome replication, and ending before the cell divides. The corresponding relationship between thermal power–time curve and the four growth phases of the *E. coli* was roughly shown in Fig. 1.

The curve A is the metabolic power–time curve recorded from *E. coli* growing in 5 mL of LB medium without addition of inhibitor, at 37.0 °C. The curve B is natural logarithm of A, which coincides with typical growth curve for a bacterial

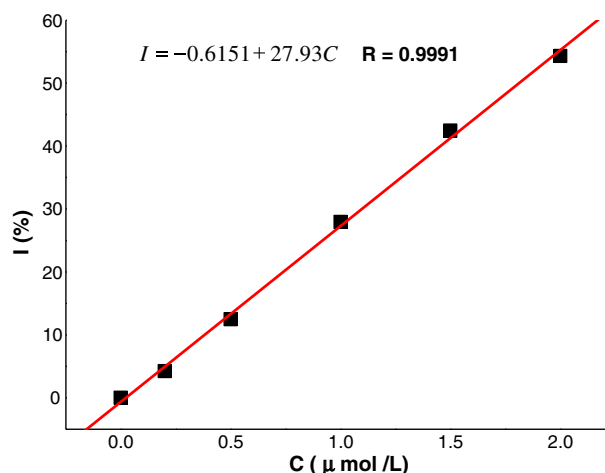
**Table 2** The half-inhibitory concentration IC<sub>50</sub> and *I*~*C* linear relationship of the inhibitors 1–7 on *E. coli* (CCTCCAB91112), the correlation coefficient *R*, and standard error SE

No.	<i>I</i> ~ <i>C</i>	IC <sub>50</sub> (μmol/L)	SE	<i>R</i>
1	$I = -2.283 + 7.254C$	7.20	0.4665	0.9793
2	$I = -0.615 + 27.93C$	1.820	0.0417	0.9974
3	$I = -1.242 + 7.742C$	6.620	0.4070	0.9814
4	$I = 2.970 + 7.107C$	6.617	0.4767	0.9756
5	$I = -1.038 + 2.641C$	19.33	1.256	0.9793
6	$I = 1.681 + 6.136C$	7.875	0.6943	0.9817
7	$I = -2.424 + 9.145C$	5.733	0.5692	0.9617

population. There is a rather slow rate of growth of a microbial culture during lag phase because the bacteria in fresh medium need time to biosynthesize essential metabolites and complete complement of enzymes for the synthesis. The heat effect is very small at the same time, and power–time curve' variability is the same. In the exponential phase, the increase in cell number is initially rather slow, but increases at an ever faster rate. In the later stages of growth, this results in an explosive increase in cell numbers. Therefore, the heat effect has a quick increase in a shorter time, and the power–time curve variability reaches the maximum. In the stationary phase, both the cell numbers in the population and the heat effect reach the maximum. The heat effect gradually decreases in pace with death of cells during death phase because of depletion of nutrition in the culture medium.

## Results and Discussion

The thermal power–time curves of the *E. coli* growth were obtained in the absence and presence of the organoarsenicals at different concentrations under controlling conditions (e.g., 37.0 °C culture temperature, 5 mL LB culture media; Fig. 2).



**Fig. 3** Relationship between inhibitory ratio and concentration of 4-(4-hydroxyl-3-methoxyl benzaliminy)phenyl arsenoxide

Through analyzing and calculating the power–time curves, we can get important thermokinetic parameters and properties of the arsenicals acting on the *E. coli* (Table 3). The thermokinetic parameters and properties including the growth rate constant  $k$ , generation time  $t_G$ , and inhibitory

ratio  $I$  (percentage) can reflect thermokinetic characteristics of the bacterial population's growth metabolism under the influence of the organoarsenicals as inhibitors. The half-inhibitory concentration  $IC_{50}$  (shown in Table 2) was afterward given by fitting a linear relationship between the

**Table 3** Thermokinetic parameters of the *E. coli* cells' growth metabolism under control (37.0 °C, 5 mL of LB liquid media) and the action of arsenical 1–7 respectively as inhibitor at different concentrations  $C$

No.	$C$ ( $\mu\text{mol L}^{-1}$ )	$K$ ( $\text{min}^{-1}$ )	$R$	$t_G$ (min)	$I$ (%)	$IC_{50}$ ( $\mu\text{mol L}^{-1}$ )
1	0	0.0352	0.9986	19.69	0	7.2
	2	0.03275	0.9986	21.16	6.96	
	4	0.02442	0.9985	28.38	30.62	
	6	0.02047	0.9987	33.42	41.85	
	8	0.01627	0.9984	42.60	52.50	
	10	0.00986	0.9982	70.30	71.98	
2	0	0.03617	0.9999	19.16	0	1.82
	0.2	0.03464	0.9997	20.10	4.23	
	0.5	0.03177	0.9971	21.82	12.16	
	1.0	0.02606	0.9964	26.60	27.95	
	1.5	0.02081	0.9952	33.31	42.46	
	2.0	0.01653	0.9940	41.93	54.30	
3	0	0.03611	0.9993	19.19	0	6.634
	1.0	0.03420	0.9990	20.26	5.296	
	1.5	0.03269	0.9995	21.20	9.471	
	2.0	0.03052	0.9989	22.71	15.48	
	3.0	0.02874	0.9986	24.12	20.41	
	4.0	0.02494	0.9870	28.13	30.93	
4	0	0.03612	0.9993	19.10	0	6.617
	1.0	0.03345	0.9986	20.70	7.418	
	2.0	0.0290	0.9963	23.90	19.73	
	4.0	0.02446	0.9934	28.30	32.30	
	5.0	0.0202	0.09960	34.31	44.07	
	10	0.0106	0.9920	65.39	70.65	
5	0	0.03726	0.9991	18.60	0	19.33
	2.0	0.03558	0.9987	19.48	4.493	
	4.0	0.03435	0.9976	20.17	7.8	
	6.0	0.03221	0.9953	21.52	13.53	
	8.0	0.02919	0.9916	23.74	21.66	
	10	0.02774	0.9903	24.98	25.54	
6	0	0.03820	0.9997	18.14	0	7.875
	1.0	0.03600	0.9996	19.25	5.759	
	1.5	0.03349	0.9988	20.70	12.33	
	2.0	0.03180	0.9991	21.80	16.75	
	3.0	0.02966	0.9959	23.37	22.36	
	4.0	0.02870	0.9934	24.15	24.87	
7	0	0.03943	0.9998	17.58	0	5.733
	1	0.03786	0.9998	18.31	3.980	
	2	0.03302	0.9991	20.99	16.26	
	3	0.03056	0.9944	22.68	22.75	
	4	0.02510	0.9958	27.62	36.34	
	10	–	–	–	–	

inhibitory ratio  $I$  (percentage) and the concentration of inhibitor (Fig. 3). It was used for tentatively evaluating the antibacterial activity of the arsenic(III)-containing Schiff bases on *E. coli* (CCTCCAB91112).

The features of metabolic power–time curves such as peak shape and peak height vary by different methods of microcalorimetry, different kinds of bacteria, and different inhibitors. The metabolic power–time curves are also called thermal spectrograms. The thermal spectrograms obtained by arsenic (III)-containing Schiff bases small molecules acting on *E. coli* (CCTCCAB91112) were given in Fig. 3. The growth thermogenic curve of the bacteria in LB media without the addition of inhibitor arsenicals possesses the maximum heat power ( $P_m$ ) and minimum time of lag phase ( $t_L$ ). The addition of the arsenicals in the LB culture media makes the growth rate and the heat production in exponential phase to decrease and the time of lag phase to increase. The higher the concentration of drugs administration was, the larger the variability of  $P_m$  and  $t_L$  became. It can be concluded that with the increase of the concentration of the inhibitor, the lag phase, i.e., the period between the beginning of the test and the ascending phase of the power–time curves, becomes longer and the maximum heat power ( $P_m$ ) decreases. From the power–time curves, it can be seen that the shapes of the metabolic thermogenic curves show almost no change at low concentrations of the arsenicals, while at higher concentrations of drug administration, the first peak becomes smaller and smaller to disappear at the end except for No. 5, and the shapes of total curves show remarkable changes with the lag phase becoming longer. It indicates that synthetic organoarsenicals arsenic (III)-containing Schiff bases have a significant inhibitory effect on the *E. coli* growth.

During the exponential phase of growth, each cell divides to form two cells, each of which also divides to form two more cells, and so on. Cells in exponential growth are typically in their healthiest state, and hence, rates of exponential growth vary greatly. From analysis of the power–time curves for *E. coli*, we can see that the thermal power production increased exponentially with increase of time during the exponential phase of growth. As described previously [28, 29], the heat production in the exponential growth phase can be expressed as follows:

$$P_t = P_0 \exp(kt) \quad (1)$$

or

$$\ln P_t = \ln P_0 + kt \quad (2)$$

where  $k$  represents the growth rate constant and  $P_0$  and  $P_t$  are the heat production of bacterial growth at the time  $t=0$  and  $t$ , respectively. Equations (1) and (2) can be applied to the thermogenic curves of the exponential growth phase to get

the growth rate constant ( $k$ ) by fitting the linear relationship  $\ln P \sim t$ . Based on the growth rate constants at different concentration of an inhibitor, the effect of the inhibitor on the bacterial growth can be quantified.

To describe the activity of arsenic (III)-containing Schiff bases on the *E. coli*, the inhibitory ratio  $I$  (percentage) can be defined as follows:

$$I(\text{percentage}) = \frac{k_0 - k_c}{k_0} \times 100 \quad (3)$$

Where  $k_0$  and  $k_c$  are the growth rate constant of the control and that in the presence of an inhibitor with a concentration of  $C$ , respectively.

We can obtain the linear relationship  $I \sim C$  via fitting  $I$  and  $C$  expressed as follows:

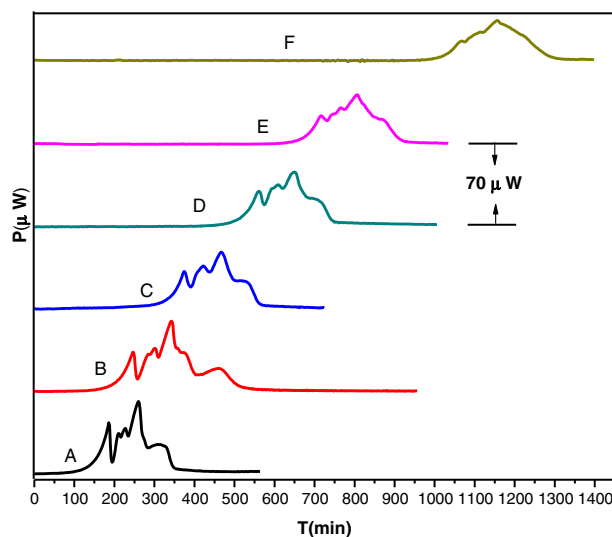
$$I = a + bC \quad (4)$$

When  $I$  equals 50, corresponding concentration of an inhibitor is the half-inhibitory concentration  $IC_{50}$ . We obtained  $IC_{50}$  of organoarsenical No. 1–7 (Table 2) for evaluating their activity on the bacteria.

The generation time ( $t_G$ ) can be obtained by the following equation:

$$t_G = \ln 2/k \quad (5)$$

In this work,  $t_G$  of the control is about 20 min. Not all *E. coli* strains replicate at 20 min. Lab strains that have been passed too many times or strains like DH5a that are fairly stripped down will have shorter generation times.



**Fig. 4** A comparison of lag phases in cell growth with drug No. 4 administration at different concentrations ( $B$  1  $\mu\text{M}$ ,  $C$  2  $\mu\text{M}$ ,  $D$  4  $\mu\text{M}$ ,  $E$  5  $\mu\text{M}$ ,  $F$  10  $\mu\text{M}$ ) and cell growth without addition of No. 4 under controlling conditions ( $A$  0  $\mu\text{M}$  of No. 4)

Through the method and principle described above, we can obtain significant thermokinetics parameters and properties (i.e., the growth rate constant  $k$ , the generation time  $t_G$ , the inhibitory ratio  $I$ , and the half-inhibitory concentration  $IC_{50}$ ) of the *E. coli* growth metabolism under control and the influence of the arsenical 1–7 as inhibitors (Table 3).

It can be seen from Table 3 that the growth rate constant decreases and the generation time and the inhibitory ratio increase with the increase of concentration of (4-(4-hydroxyl-3-methoxyl -benzaliminy)phenyl arsenoxide). The thermokinetics parameters account for the above suggest significant antibacterial activities on the *E. coli*. The half-inhibitory concentration ( $IC_{50}$ ) is widely used to evaluate the sensitivity of bacteria to inhibitors. The half-inhibitory concentration  $IC_{50}$  given in terms of Eq. (4) by fitting linear relationship of  $I \sim C$  in the Table 3 is  $1.82 \mu\text{mol L}^{-1}$ , the lowest  $IC_{50}$  and the strongest inhibitory effect on the *E. coli* among the seven synthetic compounds in this study. The functional figure of the linear equation is shown in Fig. 3:

By the same way, we can obtain the half-inhibitory concentration ( $IC_{50}$ ) of the other six arsenicals acted on the *E. coli* as shown in Table 2, and the other six functional figures of the relationship of  $I \sim C$  were similar to that in Fig. 3.

The synthesized arsenic (III)-containing Schiff bases with the exception for No. 5 exhibit considerable lag phase inhibition on the cells' growth. The administration of the arsenicals in the culture media makes the lag phase much longer than that without drug administration. When the lag phase is more than 8 h, bacterial growth is regarded as complete suppression according to traditional dose administration of three meals in one day. In the event that dose administration is not too high, many of the lag phases surpass 8 h in this study (Figs. 2 and 4). Among them, compound 4-(4-bromobenzaliminy)phenylarsenoxide has the strongest suppression on lag phase of the *E. coli* cell growth. That can be seen from No. 4 in Fig. 2, and more clearly in Fig. 4. When the concentration of No. 4 is  $1 \mu\text{M}$ , the lag phase delayed about 200 min, more than 80 min over control. The lag phase reached 650 min (about 11 h much more than 8 h) at  $5 \mu\text{M}$  of No. 4.

The result have shown that arsenic (III)-containing Schiff bases at micromolar concentration exhibited significant inhibition on the growth of *E. coli*. When the growth rate constant  $k$  decreased, the generation time  $t_G$  and the inhibitory ratio  $I$  (percentage) increased with increasing dose of the arsenicals as inhibitors. The time of lag phase of the *E. coli* population's growth cycle had a remarkable increase with the increase of drug administration dose. All but 2e displayed the feature of considerable lag phase inhibition on the cells' growth. Among them, the compounds with donating electron groups at aromatic ring B had lower  $IC_{50}$  to present higher antibacterial activity, and the lowest  $IC_{50}$

remained with 2b. The cause for this antibacterial activity is perhaps these arsenicals' inhibition on some metabolism-related key enzyme [30–32].

## Conclusions

In conclusion, this study demonstrates that microcalorimetry can provide a promising way to study the antibacterial effect of novel compounds which cannot be obtained by conventional bacteriological techniques. It also provides a convenient analytical tool for evaluating the antibacterial activity of the compounds and for screening antibacterial lead compounds. The experimental results showed that the arsenic(III)-containing Schiff bases at micromolar concentration exhibit strong inhibition on the *E. coli*. These organoarsenicals are promising to be developed into antibacterial agents with high performance. Based on this, our study direction in future will be focused on the novel organoarsenicals' effect on other microorganisms and so much as cancer cell. The toxicology and action mechanism of the organoarsenicals will be studied for further step.

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