<table>
<thead>
<tr>
<th>Title</th>
<th>Dioxiranes generated in situ from pyruvates and oxone as environmentally friendly oxidizing agents for disinfection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Wong, MK; Chan, TC; Chan, WY; Chan, WK; Vrijmoed, LLP; O'Toole, DK; Che, CM</td>
</tr>
<tr>
<td>Citation</td>
<td>Environmental Science And Technology, 2006, v. 40 n. 2, p. 625-630</td>
</tr>
<tr>
<td>Issued Date</td>
<td>2006</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10722/48513">http://hdl.handle.net/10722/48513</a></td>
</tr>
<tr>
<td>Rights</td>
<td>This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.</td>
</tr>
</tbody>
</table>
Dioxiranes Generated *in Situ* from Pyruvates and Oxone
as Environmentally Friendly Oxidizing Agents for
Disinfection

*Tak-Chung Chan, † Wai-Yee Chan, ‡ Wing-Kei Chan, † Lilian L. P. Vrijmoed, ‡ Desmond K. O'Toole, ‡
Man-Kin Wong, *† and Chi-Ming Che *†*

Department of Chemistry and Open Laboratory of Chemical Biology of the Institute of Molecular
Technology for Drug Discovery and Synthesis, The University of Hong Kong, Pokfulam Road, Hong
Kong, China

Department of Biology and Chemistry, City University of Hong Kong, Hong Kong, China

*Corresponding author phone:

(852) 2859 2154; fax: (852) 2857 1586; e-mail: cmche@hku.hk

(852) 2859 8023; fax: (852) 2857 1586; e-mail: mkwong@hkusa.hku.hk

† The University of Hong Kong

‡ City University of Hong Kong
Abstract

Dioxiranes generated in situ from pyruvates (α-ketoesters) and Oxone have been found to be environmentally friendly oxidizing agents for disinfection. These oxidizing agents were highly effective for destruction of various strains of bacteria, fungi and bacterial endospores in a wide temperature range with exceptional stability. Notably, by using an aqueous solution of methyl pyruvate (1a) and Oxone/NaHCO₃, complete destruction of bacteria such as Staphylococcus aureus and fungus Penicillium corylophilum was achieved within 5 min at 20 °C in neutral pH. Highly chemical-resistant bacterial endospores of Bacillus cereus could also be destroyed. The high antibacterial activity of 1a could be attributed to its strong electron-withdrawing α-ester group.
Introduction

Disinfection is of fundamental importance in protecting the global public health by eliminating the risk of waterborne microbial infection, controlling the outbreak of emerging infectious diseases and destruction of lethal biological weapons (1, 2, 3, 4). For more than a century, chlorine (a strong oxidizing agent) has been widely employed as the chemical agent of choice for water disinfection, microbial destruction and sewage treatment (5, 6, 7). Experimental evidences suggested that the antimicrobial action of chlorination is probably through oxidative degradation of the thiol-containing proteins/enzymes of the microbes (6). However, there are several significant drawbacks regarding the use of chlorine for disinfection. Chlorine is corrosive and may release toxic gas upon treatment with acidic solutions. In addition, chlorine-based disinfectants would generate toxic and non-biodegradable chlorinated organic compounds, some of which are known to be carcinogenic. In view of these drawbacks, it is of significant importance to develop alternative methods for disinfection (4, 5, 8, 9, 10, 11, 12). Recently, peroxide-based oxidizing agents such as Decon DF-200 (13), Decon green (14) and L-gel (15) have been introduced for microbial decontamination.

Dioxiranes, either isolated or generated in situ from ketones and Oxone, are powerful oxidizing agents capable of green organic oxidations in aqueous medium (16, 17, 18, 19, 20, 21). In particular, our previous studies have demonstrated that dioxiranes generated in situ from pyruvates (α-ketoesters) and Oxone are effective for oxidation of alkenes (22, 23), alkanes (24, 25, 26) and phenols (27). Given the significant success of dioxiranes in green oxidations, it is of interest to explore the activity of these oxidizing agents in disinfection.

Pyruvates are important metabolites widely found in living organisms: it is the end-product of glucose glycolysis and the main substrate for the Kreb’s cycle. Oxone (2KHSO$_5$·KH$_2$SO$_4$·K$_2$SO$_4$) is an inexpensive oxidant which has been used as oxidizing agent in disinfection of swimming pools (28), chemical disinfectant (29) and degradation of organic contaminants (30). Noting that pyruvates and Oxone only produce innocuous by-products including CO$_2$, H$_2$O and sodium/potassium sulfates after...
dioxirane generation. In view of their environmental friendliness, the non-toxic pyruvates and Oxone would be excellent precursors for *in situ* generation of dioxiranes.

Here we report the successful application of dioxiranes generated *in situ* from pyruvates and Oxone buffered with sodium bicarbonate as fast-acting, chlorine-free and environmentally friendly oxidizing agents for disinfection in neutral aqueous medium (Chart 1).

![Diagram of dioxirane generation](image-url)

**Chart 1.**

1a, R = CH₃
1b, R = CH₂CH₂CH₃
1c, R = CH(CH₃)₂
1e, R = H

Oxone, NaHCO₃
H₂O, pH 7
Experimental Section

Chemicals

Methyl pyruvate (1a), pyruvic acid (1e), acetone (2), Oxone and NaHCO₃ were purchased from commercial sources and used as received. α-Ketoesters 1b and 1c were prepared via acid-catalyzed esterification of pyruvic acid with n-propyl alcohol and iso-propyl alcohol, respectively, in refluxing benzene. α-Ketoester 1d was prepared by covalent attachment of pyruvic acid to β-cyclodextrin (23).

Culture of Microorganisms

(1) Preparation of bacterial suspensions of *Staphylococcus aureus* (ATCC 29737), *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 15528) and *Salmonella typhimurium* (Laboratory strain). Each bacterial strain was inoculated in nutrient broth (Oxoid) and incubated for 16 h at 37 °C. The bacteria were harvested by centrifugation at 3000 rpm for 10 minutes and washed once in sterilized quarter-strength Ringer solution (Oxoid). The bacteria pellets were resuspended in sterilized Ringer solution and the concentration was adjusted to ~ 2 x 10⁸ CFU/mL with optical density of 0.6 at 600 nm.

(2) Preparation of fungal spore suspensions of *Aspergillus niger*, *Penicillium corylophilum* and *Cladosporium sphaerospermun*. Each fungal strain was inoculated onto 2% malt extract agar (MEA) plates (20 g/L malt extract (Oxoid) + 12 g/L agar technical no.3 (Oxoid)) and incubated for 7–14 days at 25 °C. The fungal spores were harvested by addition of sterilized quarter-strength Ringer solution (with 0.05% SDS) to culture plates and scraping the plates with a spreader. The spore suspensions were transferred to a sterilized universal bottle. The concentrations of the spore suspensions were determined by using a Haemocytometer (Neubauer improved, Precicolor. W. Germany) and adjusted to ~ 2 x 10⁷ CFU/mL by appropriate dilution with sterilized quarter-strength Ringer solution (with 0.05% SDS).

(3) Preparation of bacterial endospores of *Bacillus cereus*. Cells of *Bacillus cereus* were incubated for 14–16 days at 37 °C in nutrient sporulation medium (31). The medium contained nutrient broth (Oxoid,
8 g/L), Na$_2$HPO$_4$/KH$_2$PO$_4$ buffer (67 mM Pi, pH 7.0), MgCl$_2$ (1 mM), CaCl$_2$ (0.7 mM), MnCl$_2$ (50 μM) and FeCl$_3$ (1 μM). Spores were harvested and washed once with quarter-strength Ringer solution. The spore suspension was then immersed in water bath at 60 °C for 20 min in order to destroy the remaining vegetative cells (32). The spore concentration was adjusted to ~ 2 x 10$^7$ CFU/mL with optical density of 0.7 at 600 nm.

**Bactericidal Activity Experiments of “1a / Oxone”**

A solution containing “1a (1 mM), Oxone (10 mM) and NaHCO$_3$ (31 mM)” was prepared by dissolving 1a (22 mg, 0.2 mmol), Oxone (1.23 g, 2 mmol) and NaHCO$_3$ (521 mg, 6.2 mmol) in double distilled water (200 mL). The resulting solution was sterilized by passing through a membrane filter (0.45 μm pore size), and the pH value of this solution was determined to be 7 by a pH meter. To each of the bottles containing 9 mL of the “1a / Oxone” were added a bacterial suspension (1 mL, CFU ~ 2 x 10$^8$) at 20 °C. After the designated contact time (1, 5, 15 or 30 min), three replicates of the microorganism-disinfectant solution were quenched by adding 10 mL of neutralizer solutions (0.5% w/v Na$_2$S$_2$O$_3$, 0.3% w/v lecithin and 3% w/v Tween 80 in DI water). The neutralized solutions were spread onto duplicate Nutrient Agar plates and incubated for 24 h at 37 °C. The survival of bacteria was reported as the colony-forming units per mL (CFU/mL). The initial inoculum sizes of the bacterial suspensions were further confirmed by serial dilutions with sterilized quarter-strength Ringer solution and followed by spreading on Nutrient Agar (Oxoid) plates. Colonies on each plate were counted after incubating for 24 h at 37 °C.
Fungicidal Activity Experiments of “1a / Oxone”

A solution containing “1a (5 mM), Oxone (10 mM) and NaHCO₃ (31 mM)” was prepared by dissolving 1a (110 mg, 1 mmol), Oxone (1.23 g, 2 mmol) and NaHCO₃ (521 mg, 6.2 mmol) in double distilled water (200 mL). The resulting solution was sterilized by passing through a membrane filter (0.45 μm pore size), and the pH value of this solution was determined to be 7 by using a pH meter. To each of the bottles containing 9 mL of the “1a / Oxone” solution were added a viable fungal spore suspension (1 mL, CFU ~ 2 × 10⁷) at 20 °C. After the designated contact time periods (5, 15, 30 or 60 min), three replicates of the microorganism-disinfectant solution were quenched by adding 10 mL of neutralizer solutions. The neutralized solutions were spread onto duplicate 2% malt extract agar (MEA) plates and incubated for 1–2 days at 30 °C. The survival of fungal spores was reported as the colony-forming units per mL (CFU/mL). The viability of the fungal spores was determined by a Germination test: 2% MEA agar discs of even thickness (5 mm) were cut by using the wide end of a sterilized Pasteur pipette and transferred to a sterilized Petri dish with a sterilized moisted filter paper. 2 μL of the spore suspension (~ 100 spores) was inoculated onto each agar disc. After incubating for 24 h at 30 °C, the agar discs were then fixed with lactophenol cotton blue. Using a compound light microscope, spores with germ tube were scored as germinated. In most cases, over 99% spores of all tested fungal strains could germinate within 24 h at 30 °C.

Sporicidal Activity Experiments of “1a / Oxone”

The concentration of the “1a / Oxone” solution was the same as that used in the fungicidal activity experiments. To each of the bottles containing 9 mL of the “1a / Oxone” were added a bacterial suspension (1 mL, CFU ~ 2 × 10⁷) at 20 °C. After the designated contact time periods (5, 15, 30 or 60 min), three replicates of the microorganism-disinfectant solution were quenched by adding 10 mL of neutralizer solutions. The neutralized solutions were spread onto duplicate Nutrient Agar plates and incubated for 24 h at 37 °C. The survival of bacteria was reported as the colony-forming units per mL (CFU/mL). The initial inoculum sizes of the bacterial suspensions were further confirmed by serial
dilutions with sterilized quarter-strength Ringer solution and followed by spreading on Nutrient Agar (Oxoid) plates. Colonies on each plate were counted after incubating for 24 h at 37 °C.
Results and Discussion

Antibacterial Activity

Initially, the antibacterial activity of dioxirane 1a' generated in situ from methyl pyruvate (1a) and Oxone against Staphylococcus aureus (Gram-positive bacterium) was examined. The log reduction of S. aureus with exposure time is illustrated in Figure 1. By using an aqueous solution containing 1a (1 mM), Oxone (10 mM) and NaHCO₃ (31 mM) as the antibacterial agent, 100% destruction of S. aureus could be achieved within 5 min at 20 °C in neutral pH, and 99.99% destruction (i.e. 4-log reduction count of CFU) could be achieved within 1 min. The antibacterial activity of 1a’ meets the general requirement of an efficient disinfectant that needs to achieve 99.999% killing (i.e., 5-log reduction) within 5 min at 20 °C (1).

Control experiments using a solution of “Oxone / NaHCO₃” prepared under similar conditions exhibited poor antibacterial activity, 30 min was required to achieve a 3-log reduction of CFU that is around 30-fold slower than that of “1a / Oxone”. No antibacterial activity was exhibited by 1a alone. These experiments clearly indicated that dioxirane 1a’ generated in situ from “1a / Oxone” is a highly effective antibacterial agent. By increasing the concentration of 1a to 5 mM, higher antibacterial activity (5-log reduction within 1 min) was attained (Figure 1). For comparison, a diluted household bleach (10 mM or 0.05% of OCl⁻) was found to give >5-log reduction of S. aureus in 1 min at 20 °C in neutral pH.
In general, the antimicrobial activity of chemical disinfectants would be reduced at low temperature. Yet, we are pleased to find that strong antimicrobial activity of “1a / Oxone” could be retained when the disinfection experiment was conducted at 4 °C (100% destruction within 15 min) (Figure 2). For disinfection experiments performed at 40 °C, only 5 min was required for 100% destruction.

As dioxirane 1a’ is a transient oxidizing species generated in situ from 1a and Oxone, it would be interesting to investigate the antibacterial life-time of the “1a / Oxone” solution. In this regard, three identical solutions of “1a / Oxone” were prepared and kept on shelf at 20 °C for the corresponding periods of time (2, 5 and 15 h) before disinfection experiments (Figure 3). Notably, no significant loss
of antibacterial activity (100% destruction within 5 min) was observed for the “1a / Oxone” solution prepared 2 h prior to the disinfection experiment. For the antibacterial solution prepared 5 h prior to the disinfection experiment, up to 5-log reduction in 5 min and 100% destruction in 15 min were accomplished. More importantly, after 15 h of standing the “1a / Oxone” solution was able to retain strong antibacterial activity (100% destruction within 15 min). The reasons for the long lasting antibacterial activity of “1a / Oxone” could be attributed to the high stability of 1a in the solution and the excess amount of Oxone used. As a result, sufficient concentration of 1a’ for disinfection could be maintained even after 15 h of standing.

![Figure 3](image)

**Figure 3.** Antibacterial activity of 1a (1 mM), Oxone (10 mM) and NaHCO₃ (31 mM) after standing on shelf for 2 h, 5 h or 15 h against *S. aureus* at 20 °C with different contact times (1, 5, 15, and 30 min). Each data point is the mean of three experiments ± standard deviation (CFU = Colony Forming Units).

The substituent effect of ester groups of pyruvates on the antibacterial activities was examined (Figure 4). *n*-Propyl pyruvate (1b) was found to achieve a 5-log reduction in 5 min while the analogue *iso*-propyl pyruvate (1c) could achieve a 3-log reduction in 5 min. Apart from simple pyruvates 1b–c, a β-cyclodextrin-modified pyruvate 1d (prepared by covalent attachment of pyruvic acid to a primary hydroxyl group of β-cyclodextrin) was found to afford a 3-log reduction within 5 min. Within 15 min,
*S. Aureus* could be completely destroyed by 1b−d. These results indicated that no appreciable influence on antibacterial activity was exhibited by the ester moieties. We envision that recyclable antibacterial materials could be prepared by functionalization of hydroxyl group-containing solid supports with pyruvic acid. Work in this direction is underway in our laboratory.

![Graph](image-url)

**Figure 4.** Antibacterial activity of 1−2 (1 mM), Oxone (10 mM) and NaHCO₃ (31 mM) against *S. aureus* at 20 °C at pH 7 with different contact times (1, 5, 15, and 30 min). Each data point is the mean of three experiments ± standard deviation (CFU = Colony Forming Units).

As shown in Figures 1 and 4, pyruvic acid (1e) and acetone (2) took 30 min to achieve complete destruction of *S. Aureus* that are slower than that of pyruvates 1a−d. The higher antibacterial activities of 1a−d than 1e and 2 could be attributed to the strongly electron-withdrawing α-ester groups of 1a−d (Figure 5). In the literature, it has been reported that incorporation of electron-withdrawing groups adjacent to the dioxirane functionality could enhance its activity in oxidation (33, 34). In this connection, 1a−d bearing α-ester groups may be expected to have higher oxidizing activity and hence superior antibacterial activity than unactivated ketone 2. For pyruvic acid (1e), its α-carboxylic acid...
group (pKa = 2.5) exists in equilibrium with the anionic α-carboxylate form at neutral pH. As the electron-rich ionized carboxyl group of \(1e\) is no longer electron-withdrawing, it would not enhance the oxidizing activity of the corresponding dioxirane. As a result, the antibacterial activity of \(1e\) is similar to that of 2. Our studies indicated that attachment of electron-withdrawing groups adjacent to dioxirane functionality would be the key to increase antibacterial activity of dioxirane-based oxidizing agents.

![Figure 5. Structures of 1 and 2.](image)

The “1a / Oxone” was also found to be effective for destruction of Gram-negative bacteria including *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* (Figure 6). Notably, all the three bacteria were completely destroyed within 5 min.
Figure 6. Antimicrobial activity of 1a (1 mM), Oxone (10 mM) and NaHCO₃ (31 mM) against *E. coli*, *P. aeruginosa* and *S. typhimurium* at 20 °C with different contact times (1, 5, 15, and 30 min). Each data point is the mean of three experiments ± standard deviation (CFU = Colony Forming Units).

**Antifungal Activity**

The antifungal activity of “1a / Oxone” against three common airborne fungi including *Aspergillus niger*, *Penicillium corylophilum* and *Cladosporium sphaerospermum* were also studied (Figure 7). Although spores are much more resistant toward chemical antimicrobial agents than vegetative cells (1, 2, 4), excellent antifungal activity could be obtained by increasing the concentration of 1a to 5 mM.

As depicted in Figure 7, a 4-log reduction of *A. niger* fungal spores had occurred by 15 min, and complete destruction within 60 min. Interestingly, environmental scanning electron microscopy (ESEM) revealed significant structural changes on the surface of the *A. niger* fungal spores (Figure 8). We hypothesize that the oxidizing antimicrobial agent most likely destroys the proteins/enzymes essential for germination. Note that “1a / Oxone” displayed remarkably higher antifungal activity than “Oxone / NaHCO₃” (1-log reduction in 60 min). In addition, 100% destruction of the fungal spores of *P. corylophilum* and *C. sphaerospermum* could be achieved in 5 and 15 min, respectively.

![Figure 7](image-url)

**Figure 7.** Antifungal activity of 1a (5 mM), Oxone (10 mM) and NaHCO₃ (31 mM) against fungal spores of *A. niger*, *P. corylophilum* and *C. sphaerospermum* at 20 °C at pH 7 with different contact time
(5, 15, 30 and 60 min). Each point is the mean of three experiments ± standard deviation (CFU = Colony Forming Units).

Figure 8. ESEM photographs of one of *A. niger* fungal spores (a) before and (b) after treatment with “1a / Oxone” for 60 min at 20 °C.

**Sporicidal Activity**

To demonstrate the strong antimicrobial activity of “1a / Oxone”, the sporicidal activity against highly chemical-resistant *Bacillus cereus* endospores was studied. Figure 9 shows that “1a / Oxone” was effective for reducing the numbers of the endospores (a 5-log reduction in 60 min), and further studies on the destruction of other microbes are being actively pursued.
Figure 9. Sporicidal activity of 1a (5 mM), Oxone (10 mM) and NaHCO₃ (31 mM) against endospores of *B. cereus* at 20 °C at pH 7 with different contact times (15, 30 and 60 min). Each point is the mean of three experiments ± standard deviation (CFU = Colony Forming Units).

In summary, we have developed a new class of environmentally friendly oxidizing agents for disinfection based on dioxiranes generated *in situ* from pyruvates and Oxone at neutral pH. These oxidizing agents exhibited remarkably high antimicrobial activities against vegetative bacteria, fungal spores and bacterial endospores in a wide temperature range with exceptional stability. Our results suggested that electron-withdrawing groups adjacent to the dioxirane functionality of pyruvates significantly enhance their antibacterial activity. Ongoing work is to further expand the scope of these oxidizing agents in disinfection and investigate the mechanism of their antimicrobial action.
Acknowledgements

This work was supported by the HKU Seed Funding for Applied Research, The University of Hong Kong (University Development Fund), the Hong Kong Research Grants Council (HKU7384/02P), and the Area of Excellence Scheme (AoE/P-10-01) established under the University Grants Committee (HKSAR).
Literature Cited


(13) Decon DF-200 was developed by Sandia National Laboratories (http://www.sandia.gov).


(28) http://www.dupont.com/oxone/applications/index.html

(29) http://www.antedehh.com/frameset.html


