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Note

Antimicrobial Activity of Fullerenes and Their Hydroxylated Derivatives

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The antimicrobial activities of fullerene C_{60} and its derivatives against 6 kinds of bacteria and 2 kinds of fungi were evaluated. The tested samples were water-soluble fullerenes (polyvinylpyrrolidone (PVP)/C_{60}, γ-cyclodextrin (γ-CD)/C_{60}, and nano-C_{60}) and 3 types of fullerenols ($C_{60}(OH)_{12}$, $C_{60}(OH)_{36}\cdot 8H_2O$, and $C_{60}(OH)_{44}\cdot 8H_2O$). Their activities were compared with those of (+)-catechin and hinokitiol from the viewpoint of future application to cosmetics. Although pristine C_{60} demonstrated no antimicrobial activity, fullerenols exhibited good antimicrobial activity against *Propionibacterium acnes*, *Staphylococcus epidermidis*, *Candida albicans*, and *Malassezia furfur*. In particular, $C_{60}(OH)_{44}$ exhibited a strong and wide-ranging antimicrobial activity comparable to that of catechin. This compound exhibits antimicrobial activity via inhibition of microbial cell growth and not via bactericidal activity.

Key words：Fullerenol/Antibacteria/Antifungi/MIC.

The antimicrobial activity of fullerenes and their derivatives as a new class of carbon materials has attracted significant attention in the attempt to develop novel applications in the cosmetic and pharmaceutical industries. Mashino et al. (2003) reported that alkylated C_{60}-bis (*N*,*N*-dimethylpyrrolidinium iodide) derivatives effectively inhibited bacterial cell growth. Pellarini et al. (2001) and Pantarotto et al. (2002) reported that several types of fullerene-peptides exhibited antibacterial activity against representative bacteria such as *Escherichia coli* and *Staphylococcus aureus*. On the other hand, fullerene (C_{60}) nanoparticles (particle size: less than 100 nm) did not inhibit the growth of *E. coli* (Deguchi et al., 2006). In contrast, Lyon et al. (2005, 2006) reported that these particles exhibited antimicrobial activity against *E. coli* and *Bacillus subtilis* on a cultured minimal Davis (MD) medium. Recently, Kokubo et al. (2008) have successfully synthesized highly water-soluble fullerenes (fullerenols) by using a novel hydrogen peroxide heating method. Fullerenols exhibit a superior and wide range of antioxidant activities against reactive oxygen species (ROS) by the radical scavenging activity of their allyl alcohol groups. It has been also reported that fullerenols inhibited brain cell damage by peroxide and damage of the bronchial asthma model (Tsai et al., 1997; Lai and Chiang, 1997). These studies have indicated the potential valuable applications of fullerenols. In order to investigate whether fullerenes could demonstrate antimicrobial activity, we investigated the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) of water-soluble fullerenes and their derivatives, i.e., fullerenols, against several types of bacteria and fungi.

Polyvinylpyrrolidone (PVP) entrapped C_{60} (PVP/C60, FIG. 1 (a)) and γ-cyclodextrin (γ-CD) bicapped C_{60} (γ-CD/C60, FIG. 1 (b)) were prepared

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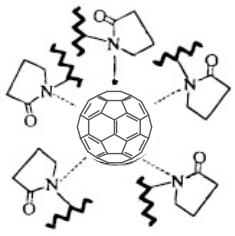
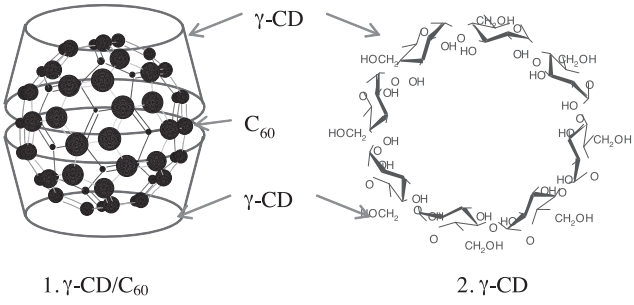
(a) PVP/C_{60}.



(b) γ-CD/C_{60}.



(c) A possible isomer of $C_{60}(OH)_{44}$.

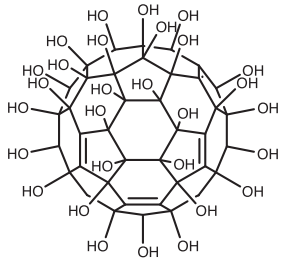


FIG. 1. Structures of water-soluble fullerene PVP/C60(a), γ-CD/C60(b), and a possible isomer of the fullerenol $C_{60}(OH)_{44}$(c).

according to the method used in previous studies (Yamakoshi et al., 1994; Andersson et al., 1992; Komatsu et al., 1999). Nano-C_{60} was produced by adapting a method from Deguchi et al. (2006). Fullerenol $C_{60}(OH)_{12}$ and fullerenol $C_{60}(OH)_{36}$ were synthesized (FIG. 2) according to the methods used in a previous study (Kokubo et al., 2008). Fullerenol $C_{60}(OH)_{44}$ was produced by modifying the fullerenol $C_{60}(OH)_{36}$ synthesis method (FIG. 1(c)).

E. coli, *Bacillus* sp., *S. aureus* (Methicillin-Resistant *Staphylococcus aureus*, MRSA), *S. aureus* (Methicillin-Sensitive *Staphylococcus aureus*, MSSA), and *Staphylococcus epidermidis* cells were inoculated into and cultured in Meuller-Hinton agar (MHA) medium at 35 ℃ for 16 to 20 h under aerobic conditions. *Propionibacterium acnes* cells were cultured in brucella agar supplemented with hemin (5

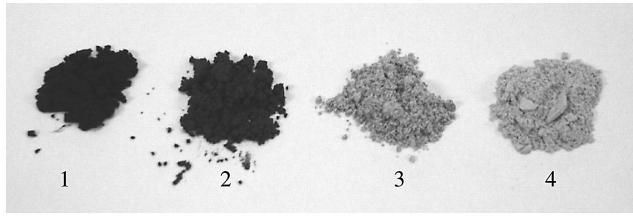


FIG. 2. Colors of fullerene C_{60} powder(1), fullerenol $C_{60}(OH)_{12}$(2), $C_{60}(OH)_{36}\cdot 8H_2O$(3), and $C_{60}(OH)_{44}\cdot 9H_2O$(4).

μg/ml), vitamin K1 (1 μg/ml), and 5% laked sheep blood at 35 ℃ for 42 to 48 h under anaerobic conditions. *Candida albicans* was cultured in RPMI1640 medium (Microbiology Systems, New York, USA) at 35 ℃ for 24 to 48 h under aerobic conditions. The *Malassezia furfur* cells were cultured in modified Leeming-Notman medium containing peptone (10 g/*l*), glucose (5 g/*l*), yeast extract (0.1 g/*l*), ox bile (4 g/*l*), glycerol (1 ml/*l*), glycerol monostearate (0.5 g/*l*), 0.5 ml Tween-60 (0.5 ml/*l*), cow's milk (whole fat; 10 ml/*l*), and Alamar blue (final concentration, 10%) at 32 ℃ for 3 d under aerobic conditions. All test samples were applied to agar plates or test tubes at 0.06 - 5120 μg/ml.

The MICs of the prepared fullerenes and fullerenols against *E. coli*, *Bacillus* sp., *S. aureus* (MRSA), *S. aureus* (MSSA), *S. epidermidis*, and *P. acnes* were determined by the agar plate dilution method according to the Clinical and Laboratory Standards Institute (CLSI) methodology (CLSI M7-A7, 2006; CLSI M11-A6, 2004; CLSI M100-S16, 2006). The MIC against *C. albicans* was determined by the minimal broth dilution assay according to the CLSI methodology (CLSI M27-A2, 2002). The MIC against *M. furfur* cultured in a modified Leeming-Notman medium was determined by the minimal broth dilution assay (Leeming et al., 1987; Garau et al., 2003; Lopez-Garcia, 2006). The MBC against *P. acnes* was determined by the method specified in the *Clinical Microbiology Procedures Handbook* (Isenberg, 2004). The MFC for *M. furfur* cultured on Dixon broth was determined by the minimal broth dilution assay (Hammer et al., 2000).

The MIC values of fullerenes against the bacteria and fungi investigated in this study are presented in Table 1. No adaptation effect was observed in any combination of fullerenes and microbial species tested. The antimicrobial activities of the well-known antimicrobial substances hinokitiol and (+)-catechin were evaluated and compared with those of fullerenes (Sawano, 1999). Hinokitiol demonstrated the strongest antimicrobial activity against all the bacteria and fungi used in this study. On the other hand, the MIC value of (+)-catechin was considerably

TABLE 1. MIC values of various fullerene derivatives (n=3).

Strain	No.	MIC (mg/L)					
		$C_{60}(OH)_{44}$	$C_{60}(OH)_{36}$	$C_{60}(OH)_{12}$	C_{60}[a]	(+)-Catechin	Hinokitiol
E. coli	ATCC 25922	−[b]	−	−	−	5120	8
	CI[c]-1	−	−	−	−	−	8
	CI-2	−	−		−	−	8
Bacillus sp.	ATCC 12432	−	−	−	−	−	8
	NBRC 13719	−	−	−	−	−	16
	ATCC 6633	−	−	−	−	5120	8
S. aureus (MRSA)	ATCC 33591	2000	−	−	−	5120	16
	CI-3	2000	−	−	−	5120	16
	CI-4	−	−	−	−	5120	16
S. aureus (MSSA)	ATCC 25923	−	−	−	−	5120	16
	CI-5	2000	−	−	−	5120	16
	CI-6	2000	−	−	−	5120	16
S. epidermidis	ATCC 12228	2000	2000	−	−	2560	0.5
	CI-7	2000	2000	−	−	2560	0.5
	CI-8	2000	2000	−	−	2560	0.5
P. acnes	ATCC 6919	1000	−	−	−	2560	64
	CI-9	2000	−	−	−	2560	64
	CI-10	−	−	−	−	2560	64
C. albicans	NBRC 1594	120	60	120	−	−	2
	CI-11	120	60	500	−	−	2
	CI-12	120	60	250	−	−	2
M. furfur	NBRC 0656	−	−	−	−	32	1
	NBRC 10987	60	120	250	−	16	1
	NBRC 10988	120	250	500	−	64	1

[a]All PVP/C_{60}, γ-CD/C_{60}, and nano-C_{60} were tested. [b]No antimicrobial activity (−) was observed.
[c]Clinical isolates in the Mitsubishi Chemical Medicine Corporation (Tokyo, Japan).

lower than that of hinokitiol. PVP/C_{60} (C_{60}=270 mg/l; Fig. 1(a)) and γ-CD/C_{60} (C_{60}=270 mg/l; FIG. 1(b)) at maximum water-soluble concentrations did not exhibit antimicrobial activity against any of the tested microorganisms. However, antimicrobial activity was exhibited by nano-C_{60} (C_{60}=500 mg/l) against all microorganisms except *E. coli*. Nano-C_{60} contained 40 mmol/l of sodium dodecyl sulfate (SDS), which was used to facilitate its dissolution in water. The MIC values of nano-C_{60} completely corresponded with those of the SDS solution; therefore, the antimicrobial activity observed was considered to be due to SDS and not nano-C_{60}. Thus, it was determined that nano-C_{60} did not exhibit antimicrobial activity. These results indicated that the water-soluble fullerenes did not possess antimicrobial activity.

On the other hand, fullerenols were observed to exhibit antimicrobial activity against several types of microorganisms. We examined the antimicrobial activity of fullerenol $C_{60}(OH)_{12}$ dissolved in 5% dimethylsulfoxide (DMSO). It was confirmed that the 5% DMSO solution of $C_{60}(OH)_{12}$ fullerenol did not show antimicrobial activity. However, $C_{60}(OH)_{44}$ (FIG. 1(c)) exhibited strong and wide-ranging antimicrobial activity comparable to that of catechin. These suggested that the hydroxyl groups of fullerenes are responsible for their antimicrobial activity. In the case of *P. acnes* ATCC 6919, the MIC value of $C_{60}(OH)_{44}$ (0.68 mol/l) was superior to that of catechin (8.8 mol/l) and comparable to that of hinokitiol (0.39 mol/l). $C_{60}(OH)_{44}$ is expected to have wide-ranging applications in the markets for household goods as well cosmetic ingredients. Further research was focused on important microorganisms such as *P. acnes* and *M. furfur* in dermatology. The MBC and MFC values of various fullerenols against each of the 10 tested strains of *P. acnes* and *M. furfur* were determined. Even at concentrations greater than 5,000 mg/l, none of the fullerenols exhibited MBC and MFC values against any of the tested strains (data not shown). This result indicates that the fullerenols exhibit antimicrobial activity through the inhibition of microbial cell growth.

Green tea extracts including catechin, nut oil extracts, and hinokitiol are known to contain antimicrobial agents, and are used for acne treatment. These agents generally possess a phenol structure with at least 1 hydroxyl group. It is believed

that the phenol structure as well as amines and alcohols contribute to the antimicrobial activity by having a disrupting effect against the bacterial cell membrane. Especially in the case of catechin, Toda and Shimamura (1996) reported that B-ring of mother nucleus-bonded hydroxyl groups played a significant role for antimicrobial and bactericidal effect. The present study also supports the idea of such a role for hydroxyl groups.

Antimicrobial activity of fullerenols against fungi was stronger than that against bacteria. Fullerenols could have a greater tendency to interact with components such as β-glucan and chitin in the fungus cell-wall than peptidoglycan in the bacterial cell-membrane. The antifungal activities slightly become strong with an increase in the water solubility of fullerenols. Therefore, the water dispersible property of fullerenols may contribute to their antifungal activity. However, the detailed antimicrobial mechanism of fullerenes and their derivatives should be clarified in order to identify their novel applications in a wide range of industrial fields.

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