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Purification and identification of an actinomycin D analogue from actinomycetes associated with *Ganoderma applanatum* via magnetic molecularly imprinted polymers and tandem mass spectrometry

Chang-gen Li[a,c,1], Bo Yuan[a,1], Meng-meng Dong[a], Pin-yu Zhou[a], Yu-xin Hao[a], Ying-ying Sun[a], Meng-ke Xu[a], Dan Li[a], Guo-yin Kai[b,**], Ji-hong Jiang[a,*]

[a] The Key Laboratory of Biotechnology for Medicinal Plants of Jiangsu Province, Jiangsu Normal University, Xuzhou, Jiangsu 221116, PR China
[b] Laboratory of Medicinal Plant Biotechnology, College of Pharmacy, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, 311402, China
[c] Yancheng Biological Engineering Higher, Xuzhou, Jiangsu 221116, PR China

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ABSTRACT

Actinomycetes are main producers of antibiotics and targeted screening could improve the efficiency of discovering new drugs. This study describes, for the first time, the isolation of endophytic actinomycetes from the macrofungus *Ganoderma applanatum*. To increase the efficiency of screening, novel actinomycin D (Act D) molecularly-imprinted polymers were adsorbed to the surface of Fe_3O_4@SiO_2 magnetic microspheres (MMIPs) and using in the isolation. A monolithic column prepared with magnetic molecularly imprinted polymers was employed to adsorb actinomycin D and its analogues for selective analysis and identification via MS/MS spectroscopy. The MMIP-monolithic column was selective for the structural features of Act D and its analogue, and the maximum loading of the MMIPs for Act D was ~23.5 μg/g. The recognition time of the Act D was 20–30 min and had good discriminative ability. A new analogue was identified from endophytic actinomycetes KLBMP 2541, and it was purified using MMIPs comparison with MMIPs-solid phase extraction. Structural identification analysis confirmed that the new analogue was 2-methyl-actinomycin D, which has better anti-tumor activity than Act D. The presented method combines the advantages of MMIPs and MS with popular solutions to enable high affinity and selectivity screening of specific antibiotics from endophytic actinomycetes.

1. Introduction

Actinomycetes are a group of Gram-positive, high G + C, and filamentous bacteria, which comprise a large part of the microbial population. Actinomycetes are widely distributed in the environment and plants (Goodfellow and Williams, 1983; Kumar et al., 2012; Ningthoujam et al., 2009). Actinomycetes (*Actinomycetales*) have been recognized as an important natural source for the bioactive antibiotics (Weber et al., 2015). Actinomycetes are also the most prolific microbial group from which the antibiotic was produced by many industrial and academic laboratories (Genilloud et al., 2011). More than half of all known bioactive antibiotics or bacterial compounds have been isolated from *Streptomyces*, which is the largest genus of actinomycetes (Bérdy, 2012). *Streptomyces* is the most important active-compounds producer in the actinomycetes, providing the largest mass of compounds with high bioactivity and commercial value. Some common antibiotics (i.e. tetracycline, erythromycin, vancomycin, and streptomycin) are the secondary metabolites of *Streptomyces* and can be obtained using isolation technology of natural products. Other natural products derived from actinomycetes, particularly *Streptomyces*, have important medical activity, such as the immunosuppressant rapamycin, doxorubicin, avermectin, and nystatin (Weber et al., 2015).

Microbial secondary metabolites are often the bioactive compounds and may have antimicrobial, antitumor, and antiviral activities, which contribute to their antibiotics function (Berdy, 2005). Actinomycins are a family of chromopeptide lactone antibiotics. All actinomycins consist of a phenoxycycl group, a chromophorous group, and two pentapeptide chains with variable amino acid composition (Kurosawa et al., 2006). Among the actinomycins, actinomycin D (Act D) is a well known antibiotic first discovered in the 1940s. It plays an important role as an anti-tumor antibiotic in many clinical trials (Hill et al., 2013). Act D is a key component in treating some tumors, such as Wilms, and the

* Corresponding author.
** Corresponding author.
E-mail addresses: boyuan@jsnu.edu.cn (B. Yuan), kaiguoyin@163.com (G.-y. Kai), jhjiang@jsnu.edu.cn (J.-h. Jiang).
[1] Yuan and Li contributed to the work equllly and should be regarded as co-first authors.

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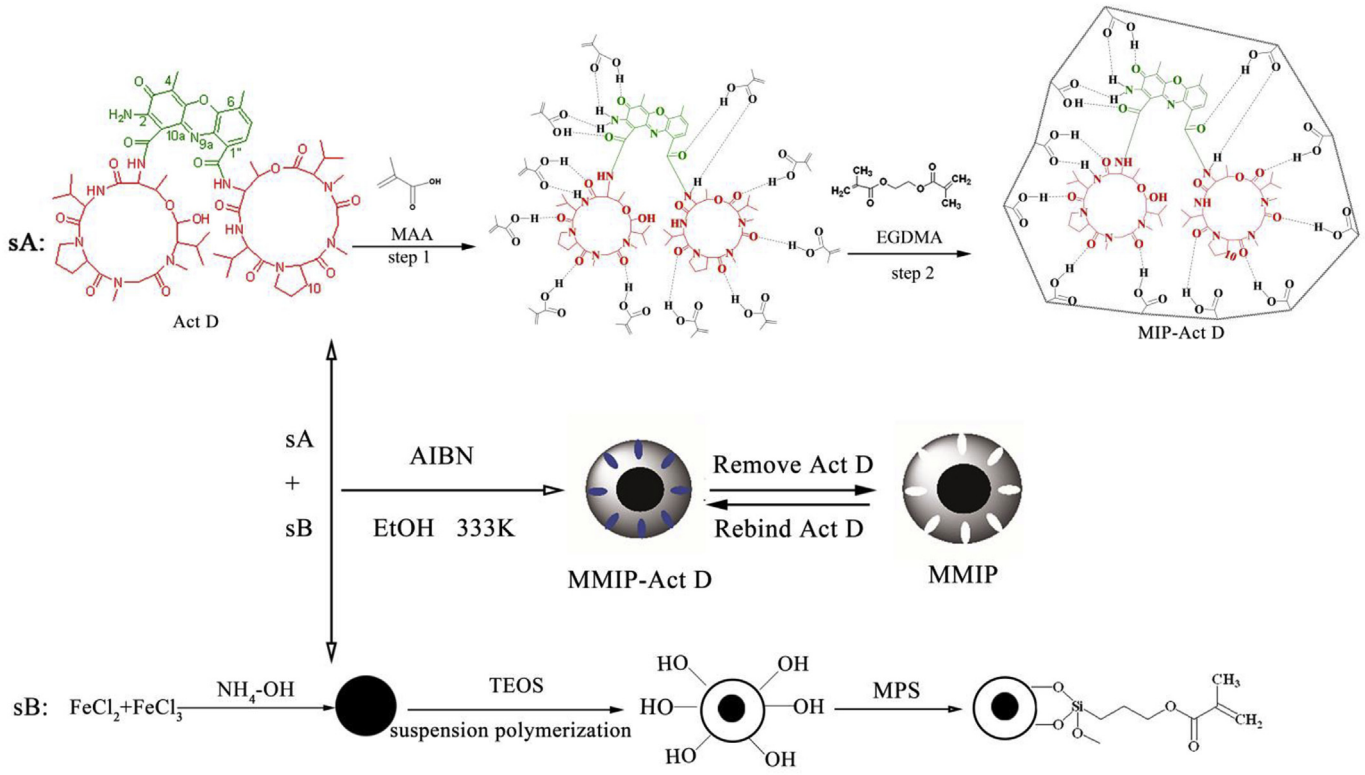
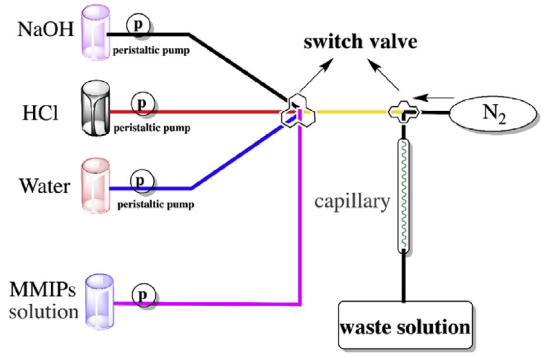


Scheme 1. Schematic of MMIP preparation.



Scheme 2. Schematic of the MMIP-monolithic column preparation.

treatment outcome can reach 80–90% in successful multidisciplinary approaches (Mann et al., 2000; Metzger and Dome, 2005). However, the toxicity and side effects of Act D are severe in practice. To minimize the side effects, a number of anti-tumor drug studies have been focused on the structural transformation of Act D. However, drug screenings for a novel natural Act D homolog or analogue have not been fully explored.

Ganoderma applanatum, a macrofungus, has been widely used in health practices in China and other East Asian countries (Bao et al., 2002). The fruiting body and spores of *G. lucidum* have been reported to have bioactive properties, including immunity enhancement, treatment of chronic hepatopathys, and anti-cancer properties. However, *G. applanatum* is difficult to find in the wild. In addition, it grows slowly and has relatively low activity under artificial cultivation. This has prompted the research community to identify similar compounds or metabolites from endophytes associated with *G. applanatum*.

Endophytes have attracted increasing attention among taxonomists, ecologists, agronomists, chemists, and evolutionary biologists as a special organism in eco-environmental microbiology (Qin et al., 2011). Endophytic actinomycetes produce an impressive array of bioactive secondary metabolites, and most of those metabolites are compounds with novel skeletons. More evidence indicates the existence of new endophytic actinomycetes within the various tissues of medicinal plants, and some of these bacteria may produce bioactive compounds with some novel chemical structures (Nimnoi et al., 2010; Passari et al., 2015; Qin et al., 2008, 2011). Recently, opportunities for discovering novel biologically active molecules from various soil actinomycetes have diminished, indicating that different environments may provide better chances for endophytic actinomycete isolation. Screening endophytic actinomycetes with antibiotic activities is an important approach in discovering new drugs targeting human and plant pathogens. A variety of methods and screening models used to target endophytic actinomycetes are important for increasing the chances of isolating novel microbial metabolites (Lee et al., 2012). Traditional methods for resistance screening (anti-tumor or bacteriostasis) involve fermentative growth and the organic extract of the metabolites. With recent advances of biotechnology, new high-throughput screening methods have been developed, including chemical genetics-based target identification and sequence-based analysis (Gontang et al., 2010; He et al., 2006).

Previously, thousands of endophytic actinomycetes have been analyzed for their bioactivities, however, the screening for special strains that produce known or unknown active compounds remains a big challenge. In this study, we aim to find additional endophytic strains with anti-tumor activity within *G. applanatum*. Molecularly-imprinted Act D polymers were adsorbed to the surface of Fe3O4@SiO2 magnetic microspheres (MMIPs). Meanwhile, a molecular engram-monolithic column was prepared for the separation of Act D and analogues from the endophytic actinomycetes extract, followed by tandem mass spectrometry (MS) analysis. The MMIP-monolithic column selectively retained Act D. In addition, a novel Act D homolog, 2-methyl-Act D, was obtained using the molecularly-imprinted polymers in conjunction with solid-phase extraction. This screening method was used to gain more specific information regarding active metabolites, and to increase the separation efficiency of Act D-like compounds from the extract of endophytic actinomycetes.