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論 文

Calorimetric Evaluation of the Antimicrobial Action of the Cosmetic Ingredient *Pionin* Dissolved in 1,3-Butanediol and 1,2-Pentanediol

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A calorimetric method was applied to the quantitative determination of the antimicrobial activity of **Pionin** — an additive used in the cosmetics industry — aspect previously investigated by other authors using more traditional methods. Because it is insoluble in the growth media, the additive required initial dissolution in 1,3-butanediol or 1,2-pentanediol — two ingredients widely used in cosmetics, which have been the subject of a previous research presented in this journal.[1,2] Based on the changes observed in the heat evolution curves corresponding to microbial cultures to which **Pionin** was added in various amounts, quantitative parameters could be determined to characterize its antimicrobial action. **Pionin** exerted remarkable antimicrobial activity against *Staphylococcus aureus* and *MRSA*, was less inhibitory against *Escherichia coli*, *Candida albicans* and *Aspergillus niger*, and had reduced antimicrobial activity against *Pseudomonas aeruginosa*. With the exception of *Candida albicans*, **Pionin** was more efficient in inhibiting microbial growth when dissolved in 1,2-pentanediol than when dissolved in 1,3-butanediol.

1. Introduction

Pionin (**Fig.1**) is a cyanine compound produced by Kankohsha Co., Ltd., which has both pigment qualities and significant antimicrobial activity when added to cosmetics in very small proportions (0.001 ~ 0.002 %). It has been described[3-6] as having a broad spectrum of antimicrobial activity, inhibiting both Gram-positive and Gram-negative bacteria, as well as yeasts and molds.

In this study, calorimetry was used for the quantitative determination of the antimicrobial effects of this cosmetic ingredient against some pathogenic microorganisms or microorganisms responsible for the spoilage of cosmetics.

2. Materials and Methods

The microorganisms studied were: *Staphylococcus aureus* 209P and MRSA OJ 51 (both from US Food and Drug Administration, Washington DC, USA); *Pseudomonas aeruginosa* (isolated from cosmetics at Kankohsha Co., Ltd.); *Candida albicans* IID 867 (obtained from the Laboratory of Culture Collections, Institute of Medical Science, Tokyo University); *Escherichia coli* IFO 3972 and *Aspergillus niger* (stock cultures of Mandom Co., Ltd, Central Research Laboratories, Osaka, Japan).

Pionin (**Fig.1**) was provided by the maker, Kankohsha Co., Ltd., Osaka, Japan.

The sample of 1,3-butanediol was a reagent grade chemical obtained from Wako Pure Chemical Industries Inc., Japan. The sample of 1,2-pentanediol was produced by Dragoco Co., Ltd, Japan.

The bacteria were cultivated at 37 ℃ in brain heart infusion broth (BHIB) obtained from Nissui Seiyaku, Tokyo, Japan. C. albicans and A. niger were grown at 30 ℃ on glucose-peptone broth (GPB) with 20 g l^{-1} glucose, provided by Wako Pure Chemical Industries

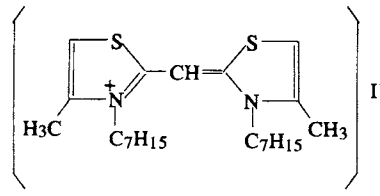
This work was conducted as a part of cooperative research programs (Project #990034) at the Center for Research and Development of Bioresources, Research Institute for Advanced Science and Technology, Osaka Prefecture Univ.

Inc., Japan. The detailed procedure for preparation of the microbial cultures was previously published.[1,2] **Pionin** needed to be dissolved in 1,3-butanediol (hereafter referred to as 1,3-BD) or in 1,2-pentanediol (hereafter referred to as 1,2-PD) before being added in various amounts to the microbial cultures, but an effort was made to keep the concentrations of these solvents as low as possible, since they have shown significant antimicrobial activity themselves.[1,2] Thus, the concentration of 1,3-BD in the microbial cultures was between 0.50 and 0.99 % w/v, while that of 1,2-PD was between 0.49 and 0.98 % w/v, depending on the microbial strain and on the need to add more or less **Pionin** to the cultures. Even in such small quantities, both 1,3-BD and 1,2-PD exert significant inhibitory action,[1] but this effect could be separated from the inhibitory effect of **Pionin**, by using control microbial cultures which contained the same amount of 1,3-BD or 1,2-PD and no **Pionin**. The concentration of **Pionin** added to the sample cultures varied between 0 and 48.3 mg l^{-1}; test experiments were carried out first in order to determine the range of concentrations most suitable to reveal the antimicrobial effect of **Pionin** against each microorganism.

Microbial growth in the presence of **Pionin** was followed using a multiplex batch calorimeter[7] that can simultaneously monitor 24 sample cultures. The working principle of the apparatus is based on the detection of the heat evolved during the growth of the microorganisms. This heat evolution in time is transformed into a voltage signal, digitized and recorded on magnetic support. The recorded data can be analyzed for the determination of the "growth rate constant μ" and another parameter called "the retardation time t_α", which in turn allow the determination of quantitative inhibitory parameters. A detailed presentation of the apparatus was made previously elsewhere.[7] At least two experiments were performed for each microorganism, which thus provided at least 48 data points for data analysis.

3. Results

Fig.2 presents some representative examples of the calorimetric curves recorded during monitoring of microbial growth in the presence of added **Pionin**. As an approximation, these curves can be considered proportional to the power evolved in the corresponding



$C_{23}H_{39}IN_2S_2$: 534.60

Fig.1 The chemical structure of **Pionin**, also known as "Kankohso 201": 2-[2-(3-heptyl-4-methyl-2-thiazolin-2-ylidene)-methine]-3-heptyl-4-methyl-thiazolium iodide.

calorimetric unit which contained a certain microbial culture. Therefore, these curves display an initial "lag" phase, when the power is below the detection limit of the apparatus, an exponentially rising portion corresponding to the exponential increase in the number of microorganisms, and a peak followed by a rapid decrease corresponding to the stop of the growth as a result of limiting conditions — nutrients, in this case. It can be seen in **Fig.2** that the addition of increasing amounts of **Pionin** to the microbial cultures led to similar changes for all microorganisms studied: as the concentration of added **Pionin** increases, the curves display a less steep exponential portion, with the peak delayed towards longer incubation times and its height decreases.

Since the procedure for data analysis of these curves has been extensively presented in previous papers,[1,7-9] for reasons of brevity only a short explanation of it will be given here. The calorimetric signals (also named $g(t)$ curves) which are presented in **Fig.2** can be integrated to calculate the actual heat evolution curves $f(t)$, which are similar and well correlated to the evolution of the actual number of microorganisms in time. With specially derived mathematic relations fitted to these curves, it is possible to calculate for each microbial culture the growth rate constant μ and the so-called "retardation time t_α" ,which represents the duration of time necessary for a culture to reach a certain level selected arbitrarily in its exponential portion. Assuming that the values of μ and t_α are μ_m and $t_\alpha(0)$ for the control culture (to which no **Pionin** was added) and μ_i and $t_\alpha(i)$ for a sample culture to which **Pionin** was added at concentration i, one can compute the values of

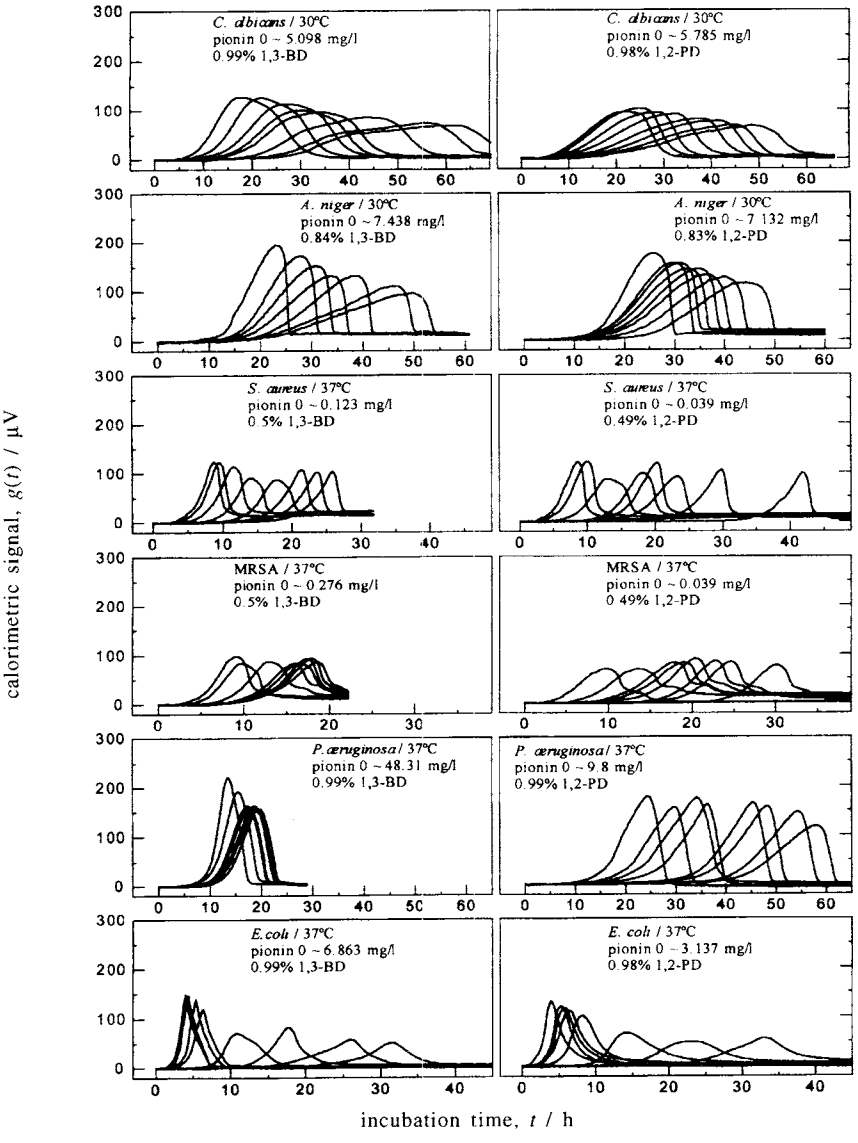


Fig.2 Representative examples of growth thermograms recorded as the calorimetric output during incubation of microbial cultures to which **Pionin** was added after having been dissolved in 1,3-butanediol or 1,2-pentanediol.

parameters μ_i/μ_m ("specific growth activity") and $t_\alpha(0)/t_\alpha(i)$ ("specific growth retardation"). These are specific parameters that may range between 0 and 1 and describe numerically the qualitative changes observed in the $g(t)$ curves in the presence of increasing amounts of **Pionin**.

The data points μ_i/μ_m and $t_\alpha(0)/t_\alpha(i)$ obtained experimentally for various values of the **Pionin** concentration i, fitted with appropriate mathematic functions,[1,2,7-9] provide the quantitative results presented in **Tables 1** and **2**.

4. Discussion

The calorimetric curves recorded during microbial growth in the presence of **Pionin** (**Fig.2**) allow some interesting observations. First of all, the general shape of the calorimetric curves, the peak height and time, the steepness of the initial rising portion can be quite

Table 1 Parameters determined calorimetrically for the characterization of the inhibitory effect of **Pionin** added to the microbial cultures. **Pionin** dissolved in 1,3-BD was added to the microbial cultures up to the maximum concentration P_{max} noted in the Table. Precision is given as the standard error.

Micro -Organism	P_{max} mg l^{-1}	1,3-BD % w/v	m_μ	K_μ mg l^{-1}	MIC$_\mu$ mg l^{-1}	m_θ	K_θ mg l^{-1}	MIC$_\theta$ mg l^{-1}
S. aureus	0.14	0.5	1.3 ± 0.2	0.16 ± 0.02	0.38 ± 0.11	1.9 ± 0.1	0.08 ± 0.01	0.18 ± 0.01
MRSA	0.28	0.5	1.4 ± 0.2	0.30 ± 0.03	0.59 ± 0.10	1.5 ± 0.2	0.22 ± 0.01	0.48 ± 0.05
E. coli	6.86	0.99	2.4 ± 0.2	3.9 ± 0.1	7.9 ± 0.4	2.6 ± 0.2	3.2 ± 0.1	7.4 ± 0.3
P. aeruginosa	48.31	2.46	0.6 ± 0.1	187 ± 45	607 ± 218	0.5 ± 0.1	388 ± 81	1447 ± 437
A. niger	8.27	0.84	1.1 ± 0.1	8.1 ± 0.7	21.1 ± 4.1	1.3 ± 0.2	6.8 ± 0.4	15.7 ± 2.0
C. albicans	4.66	0.98	0.9 ± 0.1	3.6 ± 0.2	13.5 ± 1.7	1.1 ± 0.1	3.1 ± 0.1	9.6 ± 0.6

Table 2 Parameters determined calorimetrically for the characterization of the inhibitory effect of **Pionin** added to the microbial cultures. **Pionin** dissolved in 1,2-PD was added to the microbial cultures up to the maximum concentration Pmax noted in the Table. Precision is given as the standard error.

Micro -Organism	P_{max} mg l^{-1}	1,2-BD % w/v	m_μ	K_μ mg l^{-1}	MIC$_\mu$ mg l^{-1}	m_θ	K_θ mg l^{-1}	MIC$_\theta$ mg l^{-1}
S. aureus	0.04	0.5	1.4 ± 0.3	0.04 ± 0.02	0.08 ± 0.02	2.0 ± 0.2	0.02 ± 0.001	0.05 ± 0.003
MRSA	0.04	0.5	***	***	***	1.4 ± 0.2	0.02 ± 0.001	0.07 ± 0.01
E. coli	3.43	0.98	3.7 ± 0.6	2.5 ± 0.1	3.7 ± 0.1	3.0 ± 0.3	2.0 ± 0.1	3.5 ± 0.1
P. aeruginosa	9.8	0.99	***	***	***	0.7 ± 0.06	6.8 ± 0.4	55.1 ± 15.0
A. niger	4.13	0.83	1.0 ± 0.3	4.1 ± 0.6	11.0 ± 4.1	1.4 ± 0.3	4.1 ± 0.5	8.5 ± 2.1
C. albicans	5.79	0.98	2.0 ± 0.3	7.0 ± 0.5	10.5 ± 1.2	1.4 ± 0.1	4.8 ± 0.2	10.2 ± 0.6

*** exhibits strong bactericidal activity and the parameters based on specific growth activity are not determinable.

different, depending on the microorganism involved. These are arguments supporting the possible application of calorimetry to the identificaton of microbial strains.[12]

Nevertheless, whatever the microorganism studied or the solvent employed (**Fig.2**), the calorimetric curves recorded for microbial cultures to which **Pionin** was added in increasing amounts clearly indicate a decrease in the microbial activity which, after analysis, is quantitatively expressed by the numerical results given in **Tables 1** and **2**.

The values marked by *** in **Table 2** indicate experiments in which **Pionin** displayed strong bactericidal activity, compared to the more usual case of inhibition which is a mixture of bactericidal and bacteriostatic activity. In previous papers[8-11] it was noted that, when the inhibitor has a mainly bactericidal action, the calorimetric curves recorded appear to maintain the same "steepness", irrespective of the inhibitor concentration i.[13] This in turn means that the values computed for the growth rate constant μ_i remain very close to those observed for the control cultures (μ_m), and the consequence is that it is not possible to calculate the values of the 50 % inhibitory concentration K_μ and MIC$_\mu$ based on the changes observed in the specific growth activity μ_i/μ_m. In such situations, the parameters K_θ and MIC$_\theta$ determined on the basis of the specific growth retardation $t_\alpha(0)/t_\alpha(i)$ represent the alternative which gives a quantitative indication of the antimicrobial effectiveness of the inhibitor.

In a previous report,[1] the "effective range of concentrations" was defined as the range between the smallest concentration of inhibitor which produces a visible antimicrobial effect, and the MIC value (above which it makes no sense to add more inhibitor). The

values m_μ and m_θ given in **Tables 1** and **2** represent non-dimensional parameters related to the cooperativity in the binding between the inhibitor and the living cell. The larger the values of m_μ and m_θ, the narrower is the "effective range of concentrations" along which the inhibitor displays its antimicrobial action. The most significant aspect that underlines the importance of the m_μ and m_θ parameters is this: two inhibitors which show the same value of the 50 % inhibitory concentration K_μ against a microorganism can actually have very different values of MIC_μ , if their m_μ values are very different. In conclusion, in order to completely characterize the antimicrobial activity of an inhibitor it is necessary to take into consideration all three parameters: m_μ, K_μ and MIC_μ — if the analysis is based on the specific growth activity μ_i/μ_m; m_θ , K_θ and MIC_θ — if the analysis is based on the specific growth retardation $t_\alpha(0)/t_\alpha(i)$.

Another factor which differentiates the calorimetric curves given in **Fig.2** is the solvent used for the addition of **Pionin** to the microbial cultures. In a previous report[1] it has been shown that 1,3-BD and 1,2-PD themselves have a significant antimicrobial activity. The most sensitive microorganism was *P. aeruginosa*, whose growth activity decreases by 50 % in the presence of 2.5 % w/v of 1,3-BD or 0.76 % w/v of 1,2-PD.[1] Theoretically, the independent antimicrobial effects of the solvents 1,3-BD and 1,2-PD was dismissed from analysis by using appropriate control cultures, to which no **Pionin** was added but solvents were added in the same amounts as in the sample cultures. However, there were still procedural problems especially in the case of *P. aeruginosa*, which proved to be most resistant to **Pionin** (**Tables 1, 2**). Good determination of the antimicrobial activity of **Pionin** against *P. aeruginosa* would require the addition of much larger amounts of **Pionin** to the sample cultures. However, due to the solubility limits of **Pionin** in both 1,3-BD and 1,2-PD, it would be impossible to add the required amounts of **Pionin** without causing the complete cessation of microbial growth because of the added solvent. This explains the poor precision of the 50 % inhibitory concentration and MIC values presented for *P. aeruginosa* in **Tables 1** and **2**.

If the independent antimicrobial action of the solvent is taken out of the analysis by using control cultures to which solvent was added in the same amount as in the sample cultures, it would be expected that the results which indicate the antimicrobial action of **Pionin** alone should be identical, irrespective of the solvent used. (Of course, this hypothesis should hold only when there are no synergistic or antagonistic effects involved.) Examining **Tables 1** and **2**, it appears that this situation occurred only with *C. utilis*. Although 1,3-BD and 1,2-PD have very different efficacies in inhibiting *C. utilis*,[1] when the effect of the solvent is removed from the analysis, the inhibition parameters (K_μ, K_θ, MIC_μ, MIC_θ) obtained in the presence of 1,3-BD and 1,2-PD display an irregular variation which can be considered non-significant.

In contrast, **Tables 1** and **2** show that **Pionin** was more effective in inhibiting the growth of the other 5 microorganism studied, when it was added to the culture together with 1,2-PD, than when 1,3-BD was used. This result indicates the action of a synergistic mechanism which enhances the inhibitory activity of **Pionin** in the presence of 1,2-PD, and may prove useful for the formulation of new cosmetic recipes aimed at the reduction of the content of preservatives in cosmetics.

The quantitative results given in **Tables 1** and **2** are in general agreement with data reported by other authors regarding the inhibitory efficacy of **Pionin**.[3-6] However, the present results have the advantage of being obtained with a simple and highly productive experimental procedure, which provides better precision of the calculated parameters and the possibility of revealing of synergistic and antagonistic actions. Once again, calorimetry appears to be a very suitable tool for concrete, practical studies aimed at the interactions between microrganisms and commodities in everyday use such as cosmetics and foodstuffs.

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要　旨

香粧品の原料のひとつとしてよく用いられている感光性色素ピオニンの抗微生物活性を，熱測定法により定量的に検討した。微生物培養用の培地には不溶性であるため，有機溶媒に溶解する必要があり，香粧品の原料で保湿剤として広く使用されている 1,3-ブタンジオールならびに 1,2-ペンタンジオールの少量に溶解した後，培地に添加した。ピオニンの存在下で観測された増殖サーモグラムは試験した6種のすべての微生物種について増殖抑制作用を示す特有の形状変化が認められた。得られたサーモグラムを解析し，ピオニンの微生物活性抑制パラメータを導いた。抑制効果は特に*Staphylococcus aureus* 209P，MRSA OJ 51 に対して顕著であった。一方，*Pseudomonas aeruginosa* に対する作用は，他のいずれの微生物種に対する作用よりも弱かった。溶媒についていえば，*Candida albicans* IID 867 を除き，他の全てについては1,3-ブタンジオール よりも1,2-ペンタンジオールに溶解したときの方が，抗菌作用としてはより効果的であった。

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