trained teachers to diagnose schoolchildren with malaria and treat them in primary schools was implemented in the 1990s in Tanzania, and resulted in reducing schoolchildren's absenteeism [8]. As more evidence has been reported about the influence of malaria on schoolchildren's absenteeism and cognitive performance [9,10], effective school-based approaches are anxiously awaited.

In 2000, Bundy et al. analyzed the contents of school-based malaria control programs from many perspectives [11]. Health education could promote disease prevention, because schoolchildren have come to recognize its environmental risks as well as the signs and symptoms of malaria. They have also learned to seek diagnosis and treatment. Schoolchildren could be agents for change and, indeed, it can be expected that malaria education through schools could help promote a community-wide understanding of malaria. However, the means by which the programs are introduced and implemented is not standardized; depending on the school health system of respective countries. Some programs have already included malaria education for schoolchildren as a part of community-based malaria control programs [3,12,13] and the programs were effective as a whole. However, the impact of malaria education for schoolchildren in the community has been difficult to discern from previous studies.

In this context, some organizations began implementing school-based malaria control programs around 2001 in the Greater Mekong Subregion. In Thailand, school-based malaria control programs were introduced in some limited border areas in 2001, as well. Kenan Institute Asia started "Border Action Malaria Control Program" in 25 primary schools in two provinces on the north-western border between Thailand and Myanmar in 2001. The Asian Centre of International Parasite Control (ACIPAC) also started a school-based malaria control program in one primary school in 2001 on the western border between Thailand and Myanmar. ACIPAC introduced the program to three other primary schools in the same district in 2002, and then to all the 24 primary schools in the same district in 2003. However, the effectiveness of such school-based malaria control programs, even on the schoolchildren themselves, have not yet been reported in the Greater Mekong Subregion.

The aim of this study was to evaluate ACIPAC's approach to the introduction of school-based malaria control and to describe the effectiveness of the program on schoolchildren in the target primary schools in Thailand.

2. Materials and methods

2.1. Study site

Suan Phung District, Ratchaburi Province is located on the western border between Thailand and Myanmar. Malaria is still endemic in this area. The Vector Borne Disease Control Unit 2 in Suan Phung District reported that the malaria incidence of the district was 58.2/1000 in 2001, whereas that of Thailand was 1.17/1000 in 2001 (Ministry of Public Health Thailand unpublished report 2004). The district has 24 governmental primary schools; 22 belong to the Ministry of Education and 2 belong to the Border Patrol Police Bureau, the Ministry of Interior. We introduced the program to all of the 24 schools in the district and we selected 14 of 24 schools for this study. First, we excluded seven schools; two belonged to the Border Patrol Police; four had introduced a school-based malaria control program before this study; and one was a branch school. Second, we selected 14 from the remaining 17 schools; we randomly selected three from six which had more than 200 schoolchildren, then we selected all of the remaining eleven which had less than 200 schoolchildren.

2.2. Survey method

We performed a before–after intervention study. The study was conducted from November 2003 to August 2004. After taking the baseline survey, we introduced a school-based malaria control program to the target schools in November 2003. The post survey was conducted nine months after introducing the program in August 2004.

Quantitative techniques were used for the pre- and post-surveys. The survey questionnaires were designed for 14 school principals, 111 teachers and 852 schoolchildren of grade 3, 4, 5, respectively. Of these, 14 school principals, 93 teachers and 631 schoolchildren joined the study.

The questionnaire for the school principals included questions about the context of malaria education in the school. The questionnaire for the teachers included 20 questions on their knowledge of malaria and self-evaluation of their teaching skills and teaching activities on malaria education. The questionnaire for the schoolchildren included ten questions on their knowledge of malaria, and seven on the practice malaria prevention. The questions about teachers' and schoolchildren's knowledge of malaria covered material that the Thai Ministry of Education regarded as necessary for teachers to teach about malaria and as necessary for schoolchildren to know about the disease, respectively. They regarded 70% or above correct answers as passing. The school principals and teachers filled out their questionnaires by themselves. The teachers distributed the schoolchild questionnaires to the schoolchildren who filled them out by themselves. For the post-survey, some parts of the questionnaires for the school principals and the teachers were modified. Some specific questions for the pre-survey were deleted and some specific questions for the post-survey were added, but the questions designed to compare before and after the intervention were not changed. The questionnaire for the schoolchildren was the same before and after the intervention.

This study was approved by ACIPAC's project management committee, which consists of 13 Thai and 4 Japanese representatives. For data collection, we informed the participants that we would maintain their confidentiality and anonymity. We also told them that their participation was voluntary, and that if they wished to withdraw from the study, they could leave at any point without any obligation. We then verbally obtained their consent to participate in the study.

Data were entered in the computer using SPSS (version 11.0). Statistical analysis was performed using the McNemar test.

3. The intervention

For the intervention, we provided each school with teaching manuals and schoolchildren's textbooks and held teacher training. Planning and implementation of malaria education was carried out by each school independently.

3.1. Malaria teaching materials and training

We developed a teaching manual for teachers and a textbook for schoolchildren, and held a 3-day teacher training. The teaching manual first explains basic facts about malaria. It also explains how teachers should teach about malaria to the schoolchildren. The manual includes lesson plans by grades; methods to integrate malaria education with other subjects; and examples of activities and examinations. The schoolchildren's textbook explains facts about malaria in simple language with cartoons. The basic facts about malaria cover its causes, symptoms, treatment and prevention methods.

The training was held using these materials. The school principals and one to five teachers from each school participated in the training. The number of the teachers varied according to the size of each school. After the training, the teaching manuals were distributed to all the teachers and the textbooks were distributed to all the schoolchildren of all the 17 schools.

3.2. Activities of malaria education in primary schools

After the distribution of the training and teaching manual, different schedules of malaria education were carried out at each school. Their activities are categorized as follows. 1) Lectures on the facts about malaria, 2) integration of malaria education with other subjects, such as writing essays about malaria in Thai classes, drawing pictures and singing songs about malaria in art classes, 3) outdoor activities, such as mosquito surveys, destroying breeding sites of mosquitoes, interviewing community members about malaria, 4) sending messages about malaria prevention from schoolchildren to community members by having the children produce information, education, communication (IEC) materials and bring them to their homes, issuing newsletters and posting billboards in the communities, 5) holding community events for malaria prevention by involving the parents and community members.

3.3. Expenses

It cost about US$2600 to develop, print and deliver the teaching materials. Four hundred manuals and 7000 textbooks were provided. It cost about US$2200 to hold the three-day teacher training. Twenty school principals and 53 teachers participated in the training. The total cost was about US$4800; which came to 80 cents per schoolchild. This covered not only the 17 schools in the survey but also the other 7 schools in the district. All the expenses for the activities of malaria education in primary schools were covered by schools' own recurrent budget.

4. Results

4.1. Changes in malaria education at the school level

Before introducing the malaria education program, all the schools had considered malaria to be an important issue and all had some activities for malaria prevention. However, of 14 schools, only 3 schools had their own malaria curriculum and 7 schools recorded the number of schoolchildren who contracted malaria, which was based on reports by schoolchildren or parents. In 5 schools, the teachers had discussed malaria prevention with parents but the other 9 schools had never done it. All the 14 school principals answered that the teachers occasionally told the schoolchildren to be careful not to contract malaria, however, many of them did not do any further teaching about malaria. Four schools had both teaching materials for teachers and learning materials for schoolchildren. Three schools had only teaching materials or learning materials. Seven schools had neither teaching materials nor learning materials. However, those materials were not designed for malaria education in primary schools; they were brochures for the general community distributed by hospitals or health centers.

After introducing the malaria education program, we identified several improvements. First, the teachers of 12 schools discussed malaria education and the teachers of 8 schools made their own malaria curriculum. Second, 13 of 14 schools recorded the number of schoolchildren who contracted malaria and discussed malaria prevention with parents (Table 1). Third, all the school principals answered that no teachers opposed teaching malaria in their schools, nor did any of the parents.

As a result, all the schools implemented a malaria education program in all the grades, although the activities varied school by school. In 8 schools, the teachers made their own curricula. In the other 6 schools, however, the teachers used the sample curriculum in our teaching manual to teach malaria. All the schools implemented lectures that taught malaria facts and integrated of malaria education with other subjects. Eleven schools implemented outdoor activities and twelve schools sent malaria prevention messages to community members through

Table 1
Changes in malaria education at the school level ($n=14$) (Before: November 2003; After: August 2004)

Activity	Before	After
Discuss malaria education among teachers	–[a]	12
Have own curriculum of malaria education	3	8
Record the number of schoolchildren who contracted malaria	7	13
Discuss malaria prevention with parents	5	13

[a] No data.

Table 2
Changes in malaria education at the teacher level ($n=93$)[a] (Before: November 2003; After: August 2004)

	Before	After	p value
	No. (%)	No. (%)	
Teachers' ability			
Have enough knowledge to teach about malaria ($n=91$)[b]	45 (49.5)	45 (49.5)	1.000
Can design a lesson plan on malaria ($n=88$)[b]	27 (30.7)	42 (47.7)	0.015
Teaching activity			
Have taught about malaria ($n=89$)[c]	64 (71.9)	75 (84.3)	0.035
Have integrated malaria education with other subjects ($n=87$)[c]	50 (57.5)	77 (88.5)	<0.001
Have sent messages for malaria prevention to the community through schoolchildren ($n=90$)[c]	24 (26.7)	49 (54.4)	<0.001

[a] Those who answered invalidly were excluded from respective questions.
[b] Those who answered 'yes' from 'yes', 'a little', 'little' and 'no'.
[c] Those who answered 'often' or 'sometimes' from 'often', 'sometimes', 'little' and 'never'.

schoolchildren. Three schools held community events, involving parents and community members, to appeal for malaria prevention.

4.2. Changes in malaria education at the teacher level

Before introducing the malaria education program, 45 (49.5%) teachers responded that they had enough knowledge to teach about malaria. Seventy-two (79.1%) teachers answered correctly more than 70% of the questions on knowledge of malaria. After the intervention, 82 (90.1%) teachers answered correctly more than 70% of the questions. No significant difference was found in the number of teachers who answered more than 70% of the questions correctly before and after the intervention. Statistically, there was also no significant difference in the teachers' self-evaluations of their own knowledge of malaria before and after the intervention.

Before introducing the malaria education program, 25 (28.1%) teachers had taught little or nothing about malaria, although all of them considered malaria to be an important issue in their schools. Main reasons for not teaching malaria were 'not enough knowledge' (10 teachers), 'no time' (8 teachers), 'no techniques to teach about malaria (7 teachers)', 'no curriculum' (5 teachers) and 'no materials' (4 teachers). Sixty-four (71.9%) teachers answered that they had taught about malaria often or sometimes; but only 24 (26.7%) teachers had sent messages to the community through schoolchildren.

After introducing the malaria education program, the numbers of the teachers 'who could design a lesson plan on malaria', the teachers 'who had taught about malaria', the teachers 'who had integrated malaria education with other subjects' and the teachers 'who had sent messages to the community through schoolchildren' increased with significant statistical differences. (Table 2).

4.3. Changes of schoolchildren's knowledge and behaviors

Before introducing the malaria education program, 469 (75.0%) of the schoolchildren 'had heard about malaria often or sometimes', but 156 (25.0%) of the schoolchildren answered that 'had not heard about malaria' or 'I do not know'. After introducing the malaria education program, the schoolchildren who 'had heard about malaria often or sometimes' increased to 595 (95.2%) and the schoolchildren who answered 'had not heard about malaria' or 'I do not know' decreased to 30 (4.8%). A statistically significant difference was detected before and after the intervention. However, the test scores fell even after the malaria education was implemented. Whereas 465 (73.7%) of the schoolchildren answered more than 7 of the 10 questions correctly before the intervention, 357 (56.6%) of the schoolchildren answered correctly more than 7 of the 10 after the intervention.

In 6 of 7 questions, their behaviors changed markedly, with significant statistical differences. Before introducing the malaria education program, 509 (81.8%) of the schoolchildren had always slept in mosquito nets; but the number of the schoolchildren who always took care of mosquito bites were 265 (42.7%). After introducing the malaria education program, the schoolchildren who always took care of mosquito bites increased to 385 (62.1%). The schoolchildren who always tried to go in mosquito nets as soon as possible after dinner increased from 95 (15.3%) to 121 (19.5%), but no significant difference was detected in this question. The schoolchildren who often or sometimes talked about malaria with their family, the schoolchildren who always told their family to sleep in mosquito nets when they did not do so, the schoolchildren who always reported their parents or teachers when they had fever, and the schoolchildren who always went to receive a blood examination when they suspected that they had contracted malaria also increased after the intervention (Table 3).

Table 3
Changes of schoolchildren's behaviors ($n=631$)[a] (Before: November 2003; After: August 2004)

Practice	Before	After	p value
	No. (%)	No. (%)	
Sleep in mosquito nets ($n=622$)[b]	509 (81.8)	538 (86.5)	0.014
Take care of mosquito bites ($n=620$)[b]	265 (42.7)	385 (62.1)	<0.001
Try to go in mosquito nets as soon as possible after dinner to avoid mosquito bites ($n=621$)[b]	95 (15.3)	121 (19.5)	0.051
Tell family to sleep in a mosquito net	242 (39.1)	352 (56.9)	<0.001
Tell parents or teachers soon when feverish ($n=623$)[b]	224 (36.0)	349 (56.0)	<0.001
Go to receive a blood examination when I suspected that I had contracted malaria (623)[b]	172 (27.6)	336 (53.9)	<0.001
Talk about malaria with family ($n=615$)[c]	358 (58.2)	481 (78.2)	<0.001

[a] Those who answered invalidly were excluded from respective questions.
[b] Those who answered 'always' from 'always', 'sometimes', 'no' and 'I do not know'.
[c] Those who answered 'often' or 'sometimes' from 'often', 'sometimes', 'no' and 'I do not know'.

5. Discussion

This study revealed that our school-based malaria control program was effective in changing schoolchildren's behavior with regards to malaria prevention in Thailand. Our results suggest that the keys to successful intervention lie in teacher training, with specialized malaria teaching materials, participatory learning methods, and the well-established school health system in Thailand.

First, the school principals took action to improve malaria education soon after the training and distribution of the teaching materials, and the teachers quickly started to teach about malaria. Before the training, the teachers had acknowledged the importance of malaria and had enough knowledge to teach about the disease. By training them on how they should use their knowledge to teach about malaria using the teaching materials, the teachers' attitudes towards tackling malaria in their schools were markedly improved.

After the schools actively implemented malaria education, we observed positive changes in schoolchildren's behavior towards malaria prevention. Such positive results were seen as we encouraged the teachers to use various participatory learning methods, including child-to-child approaches. The importance of these types of approaches, has been recognized. Some studies reported effectiveness of health education using child-to-child approaches [14–16]. For malaria control, it was reported that participatory learning methods were effective in changing behaviors [5].

However, schoolchildren's knowledge about malaria fell even though they came to hear about malaria more often. This might be due to a problem with the teachers' assisting the schoolchildren with the answers to the pre-survey. In the pre-survey, some teachers seemed to have led schoolchildren to the same answers when they filled out knowledge part of the questionnaire. This was suspected because almost all the answers were the same on this portion of the questionnaire in certain schools. However, this problem was not seen in the practice part of the questionnaire or in the post-survey.

As a product of schoolchildren's active participation for the program, the schoolchildren started to play important roles as messengers for malaria prevention. The teachers sent malaria prevention messages to the community through the schoolchildren by issuing newsletters or posting billboards with IEC materials made by the children themselves. The schoolchildren also reached the community through outdoor activities. The schools held community events involving parents and community members appealing for malaria prevention. These activities match the concept of WHO's health promoting school [17], which states that school health is not only for health of the schoolchildren but also health of the community. In our program, the schools indeed have involved the community in the malaria control program.

Education and collaboration with the community are important components in the struggle against malaria [13]. To achieve better results from community-based malaria control programs, improvement in the knowledge and behaviors of community members is essential. Although the schoolchildren sent malaria-related messages to their community in this program and it could be expected to bring some impact on community members, we did not survey its effect. Some previous studies have included malaria education of schoolchildren as a part of community-based malaria control program [3,12,13]. They also concluded that schoolchildren could be channels to the community but clear-cut evidence has not yet been shown in this kind of study. Further study is necessary to reveal the impact of malaria education through schools on community members.

Another challenge is how to scale-up this program to the other endemic areas of Thailand. We utilized Thailand's well-established school health system [18] and introduced the program by showing how the schools should integrate the new activities into the existing activities with the provided teaching materials. The schools accepted the program and regarded it as one of their Health Promoting School Project activities. Phinney et al. proposed that school health programs should be integrated into the curricula [19] since programs that are not based on the curricula might increase teachers' work load. It might be possible to scale-up this program to the other malaria endemic areas by utilizing the existing school health system, as long as the control program can be integrated into the curricula.

When it comes to scaling-up this effort, cost is a consideration. Our program could be an inexpensive model in Thailand. We left the planning and implementation of malaria education to each school, and simply supervised their activities. Despite such limited support from us, the schools actively implemented the malaria education program. Schoolchildren's positive behavioral changes in malaria prevention were also seen, even though the contents of their activities varied in each school. The schools started to take malaria education as one of their regular activities and implemented it with no additional budget. Thus, by utilizing Thailand's school health system, once the program is introduced, no additional budget would be required to implement malaria education itself. For expenses to introduce the program, the printing cost of the teaching materials could be reduced if the materials would be used in wider area. The training cost could also be reduced if the training for malaria education were combined with the on-going school health-related training programs.

In conclusion, our school-based malaria control program was found appropriate in Thailand. The program was effective in changing teachers' and schoolchildren's behavior, and could be effective in the wider community, although further studies are needed to confirm this. School-based malaria control could be applied to other Greater Mekong Subregion countries, as well. However, we need to be careful about the context of school health in each country, as it may be different from that in Thailand.

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第Ⅱ章 各論 検査編

1．微生物検査

1）顕微鏡検査

（4）マラリアなど原虫の検査

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竹内 勤(たけうち つとむ)*2

原虫検査の基礎

顕微鏡による原虫検査は生鮮標本や染色標本に見いだされた原虫の形態的特徴を基に，種の鑑別を行うことを基礎としている．顕微鏡検査で迅速診断が可能なヒト寄生の原虫としては，血液寄生のマラリア原虫類などの胞子虫類，トリパノソーマ類や腸管寄生のアメーバ類，鞭毛虫類，繊毛虫(大腸バランチジウム)，眼の角膜炎の原因となるアカントアメーバそして泌尿器寄生の腟トリコモナスなどがある．そのうち，「感染症の予防及び感染症の患者に対する医療に関する法律(2004年改定)」により保健所に届出が必要な原虫症としてはマラリア(四類感染症)，赤痢アメーバ症，ジアルジア症，クリプトスポリジウム症(五類感染症)がある．

試料の取り扱い

1．血液寄生原虫

1）マラリア原虫

ヒトを固有宿主とするマラリア原虫には悪性で致命率が高い熱帯熱マラリア原虫(*Plasmodium falciparum*)をはじめ，三日熱マラリア原虫(*P. vivax*)，四日熱マラリア原虫(*P. malariae*)，卵形マラリア原虫(*P. ovale*)の4種が知られている．

種の同定は，基本的にギムザ染色(Giemsa stain)した4種のマラリア原虫の形態的特徴の違いを鑑別することで行われる．血液塗抹標本作製には耳朶血などの新鮮血が適する．静脈採血する場合は，染色への影響の少ない抗凝固剤[エチレンジアミン四酢酸(ethylendiaminetetraacetic acid，EDTA)1 mg/ml]を用いる．採血後は速やかに薄層塗抹標本を作製する．少数のマラリア原虫検出を目的とする厚層塗抹法もあるが，溶血操作後の原虫の鑑別には経験を要し，一般には薄層塗抹標本を多数視野観察するほうが間違いは少ない．

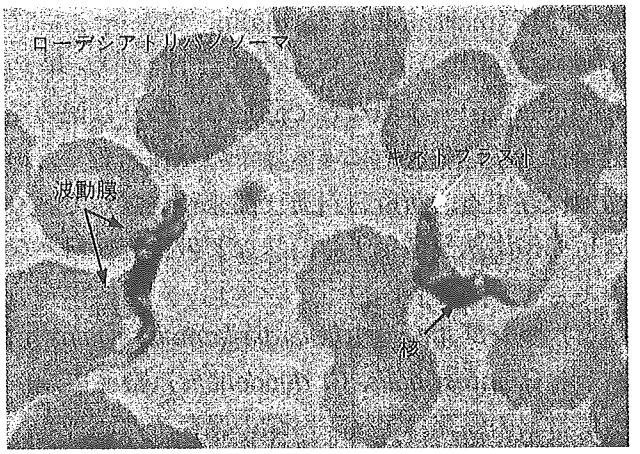

図1 ローデシアトリパノソーマ
ギムザ染色．アフリカのボツワナ共和国から帰国した女性の血液から分離された．

原虫の迅速検出法としては，蛍光色素(アクリジンオレンジ)で核酸染色する方法がある．採血は熱発作(悪寒期，灼熱期，発汗期)の時期に合わせて行うと原虫検出率が高い(発作の5～6時間後が発育した原虫が多く，種の鑑別に適すといわれる)．血液の一部を凍結保存しておくと抗原検出法や遺伝子診断法の検査材料となる．

2）アフリカ・トリパノソーマ

吸血性のツェツェバエにより媒介されるアフリカ睡眠病の病原体[ガンビアトリパノソーマ(*Trypanosoma gambiense*)，ローデシアトリパノソーマ(*T. rhodesiense*)]でツェツェバエの生息域に流行がみられる．急性期に血液，脳脊髄液などから虫体が検出される．ギムザ染色した塗抹標本で同定できる(図1)．

2．腸管寄生原虫

1）アメーバ類

ヒト腸管寄生の主なアメーバ種は病原性の赤痢

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アメーバ(*Entamoeba histolytica*)，形態的特徴が赤痢アメーバに酷似する非病原性の *E. dispar*，とハルトマンアメーバ(*E. hartmanni*)(小形種)，そして，大腸アメーバ(*E. coli*)，ヨードアメーバ(*Iodamoeba buetshlii*)，小形アメーバ(*Endolimax nana*)の6種がある．栄養型検出には，新鮮材料(便，排膿液など)を検鏡する．検査までに1晩以上を要する場合は，材料の一部をホルマリンやMF(merthiolate formalin，マーシオレイトホルマリン)液，可能であればコーン染色(Cohn stain)基本液などで固定し，残りは便の発酵に伴う発熱や腐敗による嚢子の変性を防ぐため冷蔵保存する．新鮮材料を一部凍結保存しておくと抗原検出法や遺伝子診断法などの検査材料となる．

2）ランブル鞭毛虫(*Giardia lamblia, synonym*：*G. intestinalis, G. duodenalis*)(鞭毛虫類)

便材料の保存はアメーバ類の保存法に準じる．胆汁や十二指腸液の場合は栄養型検出のため，ただちに検鏡と標本作製を行う．

3）クリプトスポリジウム，イソスポーラ，サイクロスポーラ(胞子虫類)

クリプトスポリジウム症の場合は感染力のある成熟オーシストが便に排出されるため，便の扱いには十分注意し，使用した器具も消毒(熱消毒)する．戦争イソスポーラ，サイクロスポーラの場合は1個の融合体(zygote)を有す未熟なオーシスト(oocyst)が便に排出される．外界で発育し感染力のあるスポロゾイトを包蔵する成熟オーシストとなる．便は冷蔵保存する．

3．自由生活性アメーバ類

1）アカントアメーバ

コンタクトレンズ装着者で角膜に小さな傷があると増殖し，角膜炎を起こす．角膜の擦過物を検鏡および培養法で検出する．眼科領域では直接染色法として，パーカーインクKOH法(パーカーインク1に10～20%KOH水溶液9の混合液を標本に滴下し検鏡)が汎用される．青染する嚢子(cyst)は容易に識別でき，嚢子壁(二重膜)も明瞭となる．

4．泌尿器寄生原虫類

1）膣トリコモナス

女性の場合は膣帯下，男性では尿道分泌物や前立腺分泌液を直接検鏡し，鞭毛運動する栄養型を検出する．培養法を併用すると検出率が高くなる．

測定法の概略とポイント

1．マラリア原虫

1）ギムザ染色法(Giemsa stain)

(1)塗抹，乾燥，メタノール固定(2～5分間)．

(2)3%ギムザ染色液/リン酸緩衝液(0.02 mol/*l*，pH 7.2)で40～45分間染色．緊急の場合10%ギムザ染色液で8～10分間染色する方法もある．

ギムザ染色液は，弱アルカリ性で赤血球の色調が青みを帯びるため，感染赤血球に出現する赤色のシュフナー斑点(Schueffner dot)やマウエルの斑点(Mauer dot)の識別を容易にする．水洗，乾燥，鏡検．

(3)対物油浸レンズ(100倍)で観察．赤血球内マラリア原虫は核(クロマチン核)が赤く，細胞質は青く染まる．まず，これら二つの染色性を併せもつマラリア原虫と単染する斑点を有す未熟な赤血球や血小板などとを識別し，次に確認されたマラリア原虫の種の鑑別を行う．簡単なマラリア種の鑑別点を**表1**に，ギムザ染色した熱帯熱マラリア原虫を**図2**に示す．

2）アクリジンオレンジ染色法

調製したアクリジンオレンジ液[アクリジンオレンジ(5 mg)，グリセリン(2.5 g)，10 mmol/*l* リン酸緩衝液pH 7.2～7.5(47.5 ml)]をメタノール固定した薄層塗抹部位に滴下し，カバーガラスを載せる．蛍光顕微鏡(B励起光)で検鏡する．

淡黄緑色の血球内でマラリア原虫の細胞質は橙色に，核は黄色の蛍光を示す．有核の白血球は同じ色調の強い蛍光を示す．キット化されたQBC(Quantitative Buffy Coat Systema®, Becton Dickinson)も市販されている．

3）抗原検出法

わが国では現在，NOW® MALARIA, Binax, Inc., USA/Unipath Ltd., OptiMal-IT, DiaMed AG., Switzerlandなどが実際に用いられている．凍結保存血も用いることができ，15分間程度で熱帯熱マラリア原虫とそれ以外の種との鑑別診断が可能である．

表1　簡単なマラリア原虫の鑑別表

マラリア原虫種	熱帯熱マラリア	三日熱マラリア	卵形マラリア	四日熱マラリア
被感染赤血球	膨大しない．赤色で大小不整のマウエルの斑点を見る．	膨大する．シュフナーの斑点(赤紅色の小斑)を見る．	分裂期にやや膨大．卵形，鋸歯状縁を呈す．シュフナーの斑点が全発育期に出現．	膨大しない．わずかに暗色を帯びる．不整形で薄い赤色のチーマンの斑点を稀に見る．
幼弱な栄養型(輪状体期)	小さく，2個以上の感染も普通で，2個のクロマチンを有し，血球辺縁部に付着するものが多い．	通常1個のクロマチン(赤)と大きな輪状の細胞質(青)(輪状体)を見る．重複感染は少ない．	輪状の拡がりは小さい．重複感染は稀．	輪状の拡がりは小さい．重複感染は稀．
後期栄養型	一般に，末梢血では成長した輪状体(大輪状体)まで見られる．	大きくアメーバ状を呈す．小桿状のマラリア色素を見る．	虫体は小さく，円形(卵形)のものが多く，アメーバ状を呈しない．粗大なマラリア色素を見る．	虫体は小さく，細胞質はしばしば帯状を呈す(帯状体)．粗大なマラリア色素を見る．
成熟分裂体	小さい．重症例で出現することがあるが，末梢血には稀．1つの塊状のマラリア色素を見る．(メロゾイト数：12～26)	大きい．メロゾイトも大きく，中心に集合するマラリア色素を見る．(メロゾイト数：8～24)	三日熱マラリアより小さく，マラリア色素もくすんでいる．(メロゾイト数：6～12)	メロゾイトは大きく特徴的な菊花状を呈し，中心に集合するマラリア色素を見る．(メロゾイト数：6～12)
雌雄生殖母体	雌：半月状(半月体)をなし，全体は濃青色を呈す．マラリア色素はクロマチン核を覆うように位置する．雄：両端は鈍円で雌に比べ短く，しばしば淡紅色に染まる．マラリア色素は中央に散在する．	雌：血球の全体近くを占め，偏在するクロマチン核は濃赤色に染まり，散在する粗大なマラリア色素を見る．雄：大きさは雌に近似するが，淡紅色の核は中央に位置し，細胞質は中央から遠ざかるにつれ青みを増す．	三日熱マラリアに似るが小さい．	三日熱マラリアに似るが小さく，シュフナーの斑点を欠く．

2．腸管寄生原虫

1）直接塗抹法

楊枝で採った少量の新鮮便と生理食塩水(1滴)とをスライドガラス上で混和しカバーガラスをかけて検鏡する．

下痢，軟便や粘血便ではアメーバやランブル鞭毛虫などの栄養型の特徴的な運動を見ることができる．嚢子やオーシストはその大きさ，形，色調，内容物，壁の厚さや屈光性などの特徴から鑑別を行う．アメーバや鞭毛虫類の嚢子はヨード-ヨードカリ染色を併せて行う．

ヨード-ヨードカリ液の組成と作製法は次のとおりである．ヨウ素1g，ヨウ化カリウム2g，蒸留水50 ml．ヨウ素とヨウ化カリをよく混和後，蒸留水を加えただちに振盪撹拌する．

嚢子は核の数やその性状と類染色質体やヨード胞などの特徴から種の同定を行う．核や類染色質

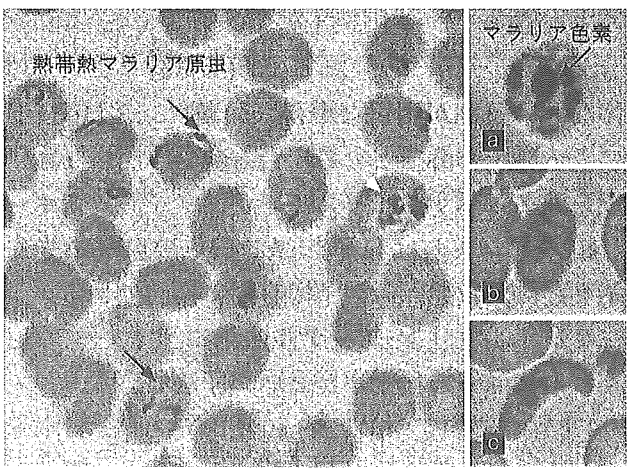

図2　熱帯熱マラリア患者(重症例)の血液塗抹標本
ギムザ染色．矢印(青)：血球辺縁部に付着する2個のクロマチン核を持つ小さなリングフォーム．矢印(赤)：成長し大きくなったリングフォーム．矢印(黄)：多重感染(三つの栄養型が見られる)．a：同じ症例の標本に見られた幼若シゾント．b(雄性生殖母体)，c(雌性生殖母体)：他の症例の治療後の患者血液標本に見られた．

表2　簡単な腸管寄生原虫類嚢子およびオーシストの鑑別表

アメーバ類(嚢子)	通常の形，大きさ(径)	核数とカリオソームの性状	類染色質体	ヨード胞の性状
赤痢アメーバ	正円(球)形(10～15 μm)	4 中心に位置し小点状	棍棒状	拡散し辺縁不明瞭
E. dispar	正円(球)形(10～15 μm)	4 中心に位置し小点状	棍棒状	拡散し辺縁不明瞭
ハルトマンアメーバ	正円(球)形(10 μm 未満)	4 中心に位置し小点状	小桿状	拡散し辺縁不明瞭
Entamoeba polecki	正円(球)形(12～14 μm)	1 分散する大小の塊状	小桿状(多数が分散)	ヨード胞に加え封入体を見る
大腸アメーバ	正円・楕円形(15～20 μm)	8 外心に位置し小点状	裂片状(稀に見る)	辺縁明瞭・大(幼若嚢子)
小形アメーバ	正円・楕円形(10 μm 未満)	4 大，球状	見られない	辺縁明瞭(幼若嚢子)
ヨードアメーバ	楕円・不整形(6～15 μm)	1 大，複数の顆粒伴う	見られない	辺縁明瞭・大きさ多様
鞭毛虫類(嚢子)	**通常の形，大きさ(径)**	**核数とカリオソームの性状**	**特徴(識別点)**	**備　考**
ランブル鞭毛虫	卵形(8～12×6～10 μm)	4 中心に位置し小点状	特徴的な三日月状の曲刺	ヨード染色し，特徴的な細胞内オルガネラを識別する
メニール鞭毛虫	洋梨形(7～9×4.5～6 μm)	1 外心に位置し小点状	大きな細胞口と鞭毛を見る	
繊毛虫類(嚢子)	**通常の形，大きさ(径)**	**核数と性状**	**特徴(識別点)**	**備　考**
大腸バランチジウム	正円(球)形(50～65 μm)	2 大核と小核を有す	腎形の大核，大きな収縮胞	嚢子の数は少ない
胞子虫類(オーシスト)	**通常の形，大きさ(径)**	**未成熟オーシスト(内容)**	**成熟オーシスト(内容)**	**備　考**
クリプトスポリジウム	短楕円形(5×4.5 μm)	成熟オーシストを排出	4個のスポロゾイトと残体	オーシスト壁は一般に他の原虫嚢子壁より薄い．未熟オーシストは浮遊法で集め，清浄な水を加えシャーレで4～6日間程度培養(25～27℃)すると発育する．
戦争イソスポーラ	長楕円形(12×30 μm)	1個の融合体を包蔵	2個のスポロシスト内に4個のスポロゾイトと残体を包蔵	
人イソスポーラ	スポロシスト(10×15 μm)(オーシスト壁失われる)	単一あるいは対の成熟スポロシストを排出	スポロシスト内に4個のスポロゾイトと残体を包蔵	
サイクロスポーラ	正円(球)形(8～10 μm)	1個の融合体を包蔵	2個のスポロシスト内に4個のスポロゾイトと残体を包蔵	

体などの識別が困難な場合，油浸レンズ(100倍)による識別が必要となる場合も多い．簡単な鑑別点を**表2**に示す．イソスポーラ，サイクロスポーラのオーシストはU励起光で青色の自家蛍光を示すため，蛍光顕微鏡下で容易に検出できる．

2）ホルマリン-エーテル法

原虫類嚢子の検出率が高い集卵法である．

(1)試験管(15 ml)に約10 mlの生理食塩水を加え，これに割り箸などで採った大豆大の糞便を入れよく撹拌する．

(2)漏斗にガーゼを1，2枚敷き遠沈管(10 ml)に濾過する．

(3)1,500 rpmで5分間遠沈し，上清を捨て，6～7 mlの10%ホルマリンを加え撹拌し15分間以上固定する．

(4)ジエチルエーテルを2～3 ml加え，密栓し指で押さえ強く振盪撹拌する．栓を取り1,500 rpmで5分間遠沈する．エーテル層の糞便質を楊枝で管壁から切り放し上清を捨てる．付着する糞便質は綿棒で拭い取る．

(5)沈渣を直接，あるいはヨード-ヨードカリ染色し検鏡する．

3）ショ糖浮遊法

クリプトスポリジウムのオーシスト検出に用いられる．

簡易迅速ショ糖浮遊法は次のように行う．

シーターのショ糖液(Sheather's sucrose solution)[ショ糖(スクロース)100 g，蒸留水64 ml，液状フェノール1 ml(比重約1.3)]をスライドガラス上に取り，ショ糖液の1/3程度の下痢便を加

え，カバーガラスの角で撹拌する．カバーガラスを載せ5分間静置した後，ピントをカバーガラスの直下の液面に合わせ，浮上してきたクリプトスポリジウムのオーシスト(長径約5 μm)を検出する．

オーシストは屈光性の違いから背景より明るく，白からピンク色に見える．

ショ糖遠心浮遊法のほうが検出率は高い．

4）コーン染色法

アメーバや鞭毛虫の種の鑑別のために用いられる．コーン染色液の組成は次のとおり．

基本液：90%エタノール(170 ml)，100%メタノール(160 ml)，酢酸(20 ml)，液状石炭酸(20 ml)，1%リンタングステン酸(12 ml)，蒸留水(618 ml)/1,000 ml

クロラゾール・ブラックE溶液：クロラゾール・ブラックE粉末(5 g)，基本液(1,000 ml)

コーン染色液で固定と染色を同時に行う．筆者らはカバーガラスに便を塗抹し，塗抹面を下にして表面張力を利用して染色液面に浮かせ，固定，染色を行っている．材料を基本液に保存しておくと，後にコーン染色やトリクローム染色を行うことができる．

5）抗酸染色

(1)糞便を塗抹，乾燥，メタノール固定2～5分間．

(2)石炭酸フクシン液[チール(Ziehl)氏カルボールフクシン液(武藤化学薬品)]で染色(5～10分間)．

(3)水洗．

(4)5%硫酸水で脱色(5～10秒間)．

(5)水洗．水洗後，塗抹面の赤色が消える程度がよい．

(6)1%メチレンブルー水溶液で後染色(1分間)．

(7)水洗，乾燥．

(8)キシレンで透徹後，バルサム封入．オーシスト内部の構造物がピンクから濃赤色に染まる．

FITC(fluorescein isothiocyanate，フルオレセインイソチオシアネート)標識した抗クリプトスポリジウム・オーシスト壁モノクローナル抗体を用い，オーシストを検出する直接蛍光抗体法のキット[Merifluor Crypto & Giardia(Meridian Bioscience Inc.)]やクリプトスポリジウム検出キット(和光純薬)も市販されている．

6）抗原検出法

赤痢アメーバ，クリプトスポリジウムやランブル鞭毛虫の特異抗原検出キット[*E. histolytica II* や *Cryptosporidium* Test(TechLab)，Immunocard STAT Crypto/Giardia(Meridian Bioscience)]などが市販されている．

検査上の留意事項，検体の選定法

感染が考えられる原虫種の性質を理解し，検体の選定と迅速な検査を行う．陰性の場合は他の検査法にも留意する．

1．結果の解釈

1）検査結果の読みかた

熱帯熱マラリアの場合，赤血球へのマラリア原虫の感染率[百分率(%)]が，重症度の指標になる．免疫のない日本人では1%を超えると多くは重症になる．

2）異常所見と参考事例

(1)抗マラリア薬の服薬により，マラリア原虫の形態や染色性が異常所見を示すことがある．

(2)下剤投与により，アメーバ(栄養型)の萎縮が起こり，生検材料(病理組織切片標本)のアメーバの識別が困難となった例がある．

(3)泌尿器以外の口腔膿瘍から腟トリコモナスが検出された症例や，ウシのトリコモナス(*Trichomonas foetus*)が，免疫不全のヒトの髄液や尿から検出された症例もある．

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O-Benzyl-*N*-*tert*-butoxycarbonyl-L-threonyl-L-proline trichloroethyl ester [Boc-L-Thr(Bzl)-L-Pro-OTce]

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Key indicators

Single-crystal X-ray study
T = 173 K
Mean σ(C–C) = 0.009 Å
R factor = 0.063
wR factor = 0.145
Data-to-parameter ratio = 13.7

For details of how these key indicators were automatically derived from the article, see http://journals.iucr.org/e.

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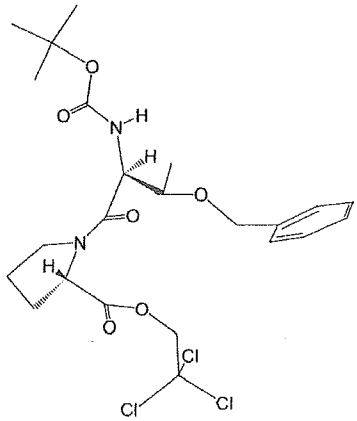
The title peptide compound, $C_{23}H_{31}Cl_3N_2O_6$, is a synthetic intermediate as a plasmodium falciparum blood-stage antigen. There is an intramolecular N—H···O hydrogen bond between the urethane and benzyl ether groups. The relatively low melting point is attributed to the lack of an intermolecular hydrogen-bond network.

Comment

The title compound, (I) is a key starting material (Omi *et al.*, 2005) in our continuing studies of synthetic antigens for falciparum malaria (Karasawa *et al.*, 2000; Ishiguro *et al.*, 2001; Kokubo *et al.*, 2002; Noi *et al.*, 2003.



Boc-L-Thr(Bzl)-L-Pro-OTce
(I)

Generally, in peptide synthesis, the 2,2,2-trichloroethyl group (—OTce) is useful for carboxyl protection and can be removed simply by treating the peptide with zinc powder in acetic acid (Marinier *et al.*, 1973; Olsen *et al.*, 1986; Pastuszak *et al.*, 1982; Yamada *et al.*, 2003; Endo *et al.*, 2003; Oku *et al.*, 2005). We often encounter oily products and poor crystallinity when we prepare *N*-protected peptide trichloroethyl esters, such as *Z*-Ala-OTce (Dhaon *et al.*, 1982), *Z*-Leu-Ala-OTce (Marinier *et al.*, 1973), Boc-Val-Leu-OTce (Yamada *et al.*, 2003) and Boc-Asp(OBzl)-Leu-OTce (Omi *et al.*, 2005). Therefore, in this paper, to assess the enantiopurity and crystallinity, we have studied the solid-state structure of (I) by X-ray crystallography.

There is one molecule in the asymmetric unit (Fig. 1). An N—H···O hydrogen bond (Table 2) is found between O202 of the benzyl ether and N201—H1 of the urethane group. There is no intermolecular hydrogen bond and molecules are probably connected together by van der Waals forces and dipole–dipole interactions (Fig. 2). The relatively low melting point of

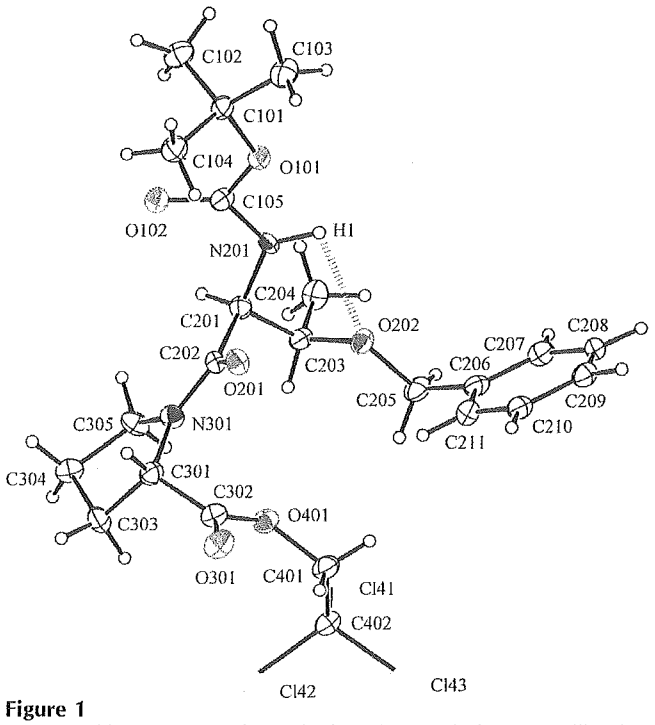


Figure 1
A view of (I) with the atomic numbering scheme. Displacement ellipsoids are drawn at the 20% probability level. The dashed line indicates a hydrogen bond.

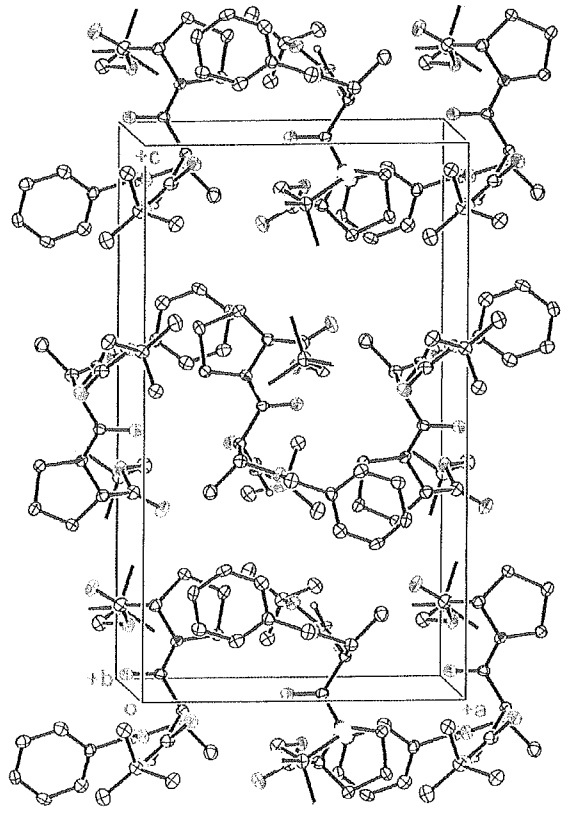


Figure 2
A packing diagram of (I). H atoms have been omitted for clarity, except for those of NH groups.

(I) is attributed to the lack of an intermolecular hydrogen-bond network, which is an important crystallizing force for short-peptide compounds (Oku *et al.*, 2003, 2003*a*,*b*; Antolic *et al.*, 1999; Ashida *et al.*, 1981; Cruse *et al.*, 1982). Thus, the weak intermolecular association in the crystal structure and the thermal mobility, especially at Boc-Thr(Bzl), probably lowers the melting point of (I).

Experimental

The title compound, (I), was prepared by the coupling of Boc-Thr(Bzl)-OH (5.10 g, 16.5 mmol) and HCl·Pro-OTce (4.24 g, 15.0 mmol) as a solution-phase synthesis. Dicyclohexylcarbodiimide (3.41 g, 15.0 mmol) was used as a coupling reagent (yield 6.55 g, 81%). Crystals of (I) were successfully grown from an oil by the addition of diethyl ether or n-hexane and stored below 277 K overnight. The fine platelets have shown relatively low melting point, 381–382 K. Analytical data (melting point, ^{1}H NMR, ESI–MS and $[\alpha]_D^{20}$) are in accordance with the expected structure; $[\alpha]_D^{20} = -49.4°$ (*c* 0.1, methanol).

Crystal data

$C_{23}H_{31}Cl_3N_2O_6$
$M_r = 537.87$
Orthorhombic, $P2_12_12_1$
$a = 11.311$ (9) Å
$b = 11.693$ (7) Å
$c = 19.417$ (12) Å
$V = 2568$ (3) Å^3
$Z = 4$
$D_x = 1.391$ Mg m^{-3}
Cu $K\alpha$ radiation
Cell parameters from 20848 reflections
$\theta = 3.9$–$67.2°$
$\mu = 3.58$ mm^{-1}
$T = 173.1$ K
Platelet, colorless
0.05 × 0.02 × 0.01 mm

Data collection

Rigaku R-AXIS RAPID diffractometer
ω scans
20848 measured reflections
4647 independent reflections
2140 reflections with $F^2 > 2\sigma(F^2)$
$R_{int} = 0.071$
$\theta_{max} = 68.2°$
$h = -13 \rightarrow 13$
$k = -14 \rightarrow 14$
$l = -23 \rightarrow 23$

Refinement

Refinement on F^2
$R[F^2 > 2\sigma(F^2)] = 0.063$
$wR(F^2) = 0.145$
$S = 0.97$
4647 reflections
339 parameters
H-atom parameters constrained
$w = 4F_o^2/[0.0008F_o^2 + \sigma(F_o^2)]$
$(\Delta/\sigma)_{max} < 0.001$
$\Delta\rho_{max} = 0.74$ e Å^{-3}
$\Delta\rho_{min} = -1.01$ e Å^{-3}
Absolute structure: Flack (1983), 1983 Friedel pairs
Flack parameter: 0.16 (2)

Table 1
Selected geometric parameters (°).

C101—O101—C105—N201	−177.5 (5)	C202—N301—C301—C302	−63.5 (7)
C401—O401—C302—C301	−172.5 (5)	C301—N301—C202—C201	174.6 (5)
C105—N201—C201—C202	−68.8 (6)	N201—C201—C202—N301	164.0 (5)
C201—N201—C105—O101	162.6 (5)	N301—C301—C302—O401	−33.3 (7)

Table 2
Hydrogen-bond geometry (Å, °).

D—H···*A*	*D*—H	H···*A*	*D*···*A*	*D*—H···*A*
N201—H1···O202	0.95	2.25	2.601 (6)	101

H atoms were positioned geometrically and refined using a riding model, with N—H = C—H = 0.95Å and U_{iso}(H) = 1.2U_{eq}(N,C). The absolute configuration of (I) agrees with the fact that the ^{1}H NMR spectroscopic data detected no racemization in the preparation.

Data collection: *RAPID-AUTO* (Rigaku/MSC, 2003); cell refinement: *RAPID-AUTO*; data reduction: *CrystalStructure* (Rigaku/MSC, 2003); program(s) used to solve structure: *SIR2002* (Burla *et al.*, 2003); program(s) used to refine structure: *CRYSTALS* (Betteridge *et al.*, 2003); molecular graphics: *ORTEP* (Johnson, 1965); software used to prepare material for publication: *CrystalStructure*.

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Synthesis of Sequential Polydepsipeptide Microspheres as a Controlled Drug Delivery System

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Sequential polydepsipeptides poly(X^1-X^2-X^3-Y) (X=amino acid, Y=hydroxy acid) were prepared by solution-phase procedure for peptide to apply for the biodegradable microspheres. Polydepsipeptide microspheres encapsulating bovine serum albumin (BSA) were prepared by a double emulsion method [1]. These microspheres were incubated in a phosphate-buffered saline and investigated the protein release profiles.

Keywords: polydepsipeptide, solution-phase synthesis, bovine serum albumin controlled release, microsphere.

Introduction

Biodegradable microspheres as a drug delivery system have been extensively investigated. Most of microspheres are being made of poly(lactic acid) (PLA) or poly(lactic-co-glycolic acid) (PLGA). These polymers, however often cause the inflammation of the affected part due to their acidity, and the control of release of included drags is difficult [2]. To improve the disadvantages of these microspheres, we studies sequential polydepsipeptides poly(AA^1-AA^2-AA^3-HA) (AA=amino acid, HA=hydroxy acid). Polydepsipeptides are biodegradable, and highly biocompatible. Furthermore, the degradation of the polydepsipeptides can be successfully controlled by changing amino acids and hydroxy acids [3]. Therefore, there is a possibility that the ideal microsphere could be achieved by using polydepsipeptides.

In this study, we prepared some sequential polydepsipeptides from tetradepsipeptide active-esters as a monomer obtained by solution-phase peptide synthesis (Fig. 1).

The polydepsipeptide microspheres encapsulating BSA were prepared by double emulsion method. Release of the protein *in vitro* was analyzed.

Results and Discussion

The monomer was prepared by stepwise elongation of peptide chains from a hydroxy acid benzyl ester by using *N,N'*-dicyclohexylcarbodiimide (DCC). The intermediates and monomers were highly purified by using column chromatography or recrystallization. The monomers were dissolved at a high concentration in a solvent and polymerized by addition of triethylamine with vigorous stirring. The stirring was

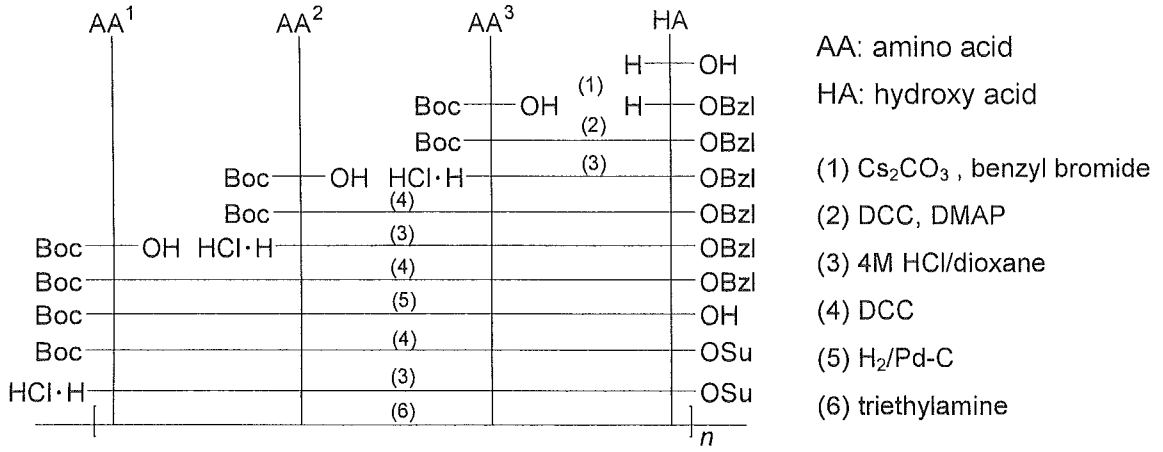

Figure 1. Synthetic procedure of polydepsipeptides.

continued for two days at room temperature. Polydepsipeptides were precipitated by distilled water and filtered.

The physicochemical properties of the polymers were dramatically changed with variation of the depsipeptide sequence. (Table 1). The polydepsipeptide containing lysine residues was highly viscous.

Polydepsipeptide microspheres were prepared by a double emulsion method (Fig. 2). Ten mg of the protein was dissolved in 150μL of distilled water and the solution was poured into a solution of 0.1 g polydepsipeptide in 1.5 mL methylene chloride. The mixture was sonicated for 3 min to form first inner emulsion (w/o). The emulsion was poured into 15mL of (0.5, 2.0, and 5.0%) PVA aqueous solution and sonicated for 5 min to form second emulsion (w/o/w). The resulting double emulsion was continuously stirred for 6 h at room temperature until methylene chloride was evaporated out. The microspheres were collected by centrifugation at 5,000 rpm for 30 min (HITACHI, 05P-22, Japan) and freeze-dried for 48h (IWAKI, FRD-50M, Japan) to give a powder.

We tried to prepare microspheres of poly[Glu(OEt)AlaAlaHmp] and poly[AlaLeuLys(Z)Lac], but failed to obtain the microspheres from the former polymer, probably because the viscosity of the polymer was too low to form the microspheres.

Table 1. Physicochemical data of polydepsipeptides.

polydepsipeptide	solvent	m.p. (°C)	viscosity[a] (dL/g)
$[LeuLeuLeuLac]_n$	DMF	145-158	0.22
$[AlaLeuLys(Z)Lac]_n$	DMSO	147-165	0.65
$[AlaAlaGlu(OEt)Lac]_n$	DMF	275-280	0.26
$[Glu(OEt)Glu(OEt)Glu(OEt)Lac]_n$	DMF	245-250	0.24
$[AlaAlaGlu(OEt)Hmp]_n$	DMF	271-279	0.31
$[Glu(OEt)AlaAlaHmp]_n$ (1)	DMF	240-250	0.23
$[Glu(OEt)AlaAlaHmp]_n$ (2)	DMSO	219-227	0.23
$[LeuGlu(OEt)AlaHmp]_n$	DMF	109-116	0.14

a) in dichloroacetic acid , c 0.5

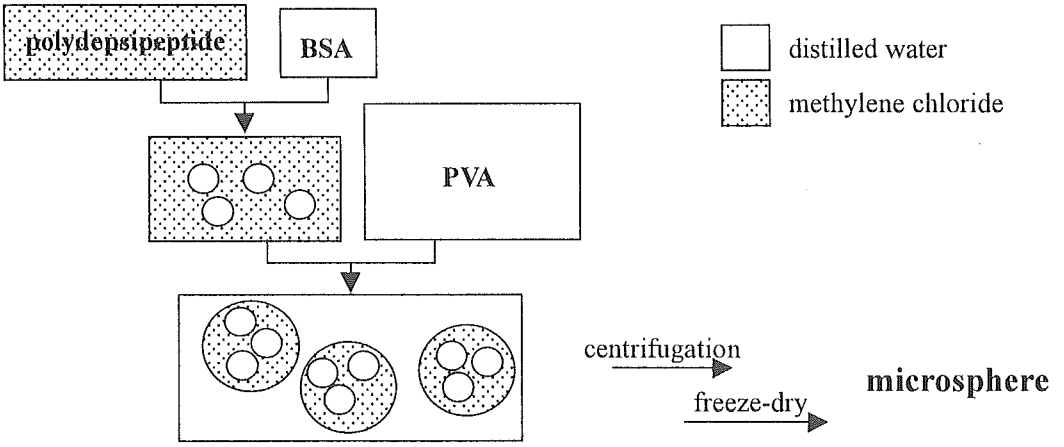

Figure 2. Double emulsion method used for the preparation of BSA-loaded polydepsipeptide microspheres.

SEM images of microspheres at different concentrations of PVA were shown in Fig. 3. When 5.0% PVA aqueous solution was used, it was not uniform in size. We suppose that the viscosity might be too high to form the homogeneous emulsion.

Loading efficiency was determined by analyzing the BSA that was not encapsulated by using the BCA protein assay kit. Effect of the concentration of PVA in the preparation of microspheres to the sphere size and loading efficiency of BSA is shown in Table 2. Loading efficiency was the highest at 0.5% concentration of PVA. The changes in the size of microspheres and efficiency of loading of BSA depended not only on the PVA concentration but also the supersonic conditions.

Finally, the *in vitro* release of BSA from the microspheres was studied (Fig. 4). Microspheres (10 mg) were suspended in 1 mL of a phosphate-buffered saline. The suspension was placed in an incubator at 37°C and continuously shaken gently. A part of the sample was periodically taken, centrifuged for 10 min at 12,000 rpm, and the supernatant was analyzed to give the amount of released BSA. Every sphere showed a similar biphasic release profiles with an initial fast release phase and a second slow release phase. The amount of protein released from the microsphere using 2.0% PVA was found to be highest. It is not clean yet whether the *in vitro* release of encapsulated protein strictly correlates with the PVA concentration.

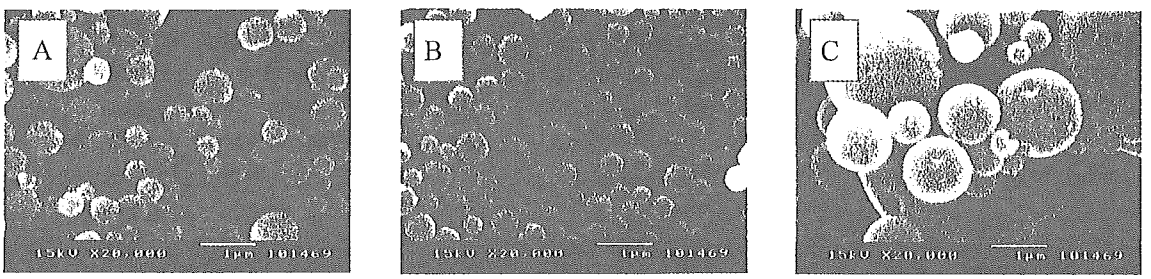

Figure 3. SEM images of microspheres at the different concentration of PVA. (A) 0.5%; (B) 2.0%; (C) 5.0% PVA aqueous solution was used for preparation of microspheres.

Table 2. Characterization of BSA-loaded microspheres.

PVA conc. (%)	sphere size (nm)	loading efficiency (%)
0.5	440±150	65
2.0	450±130	38
5.0	1470±1230	40

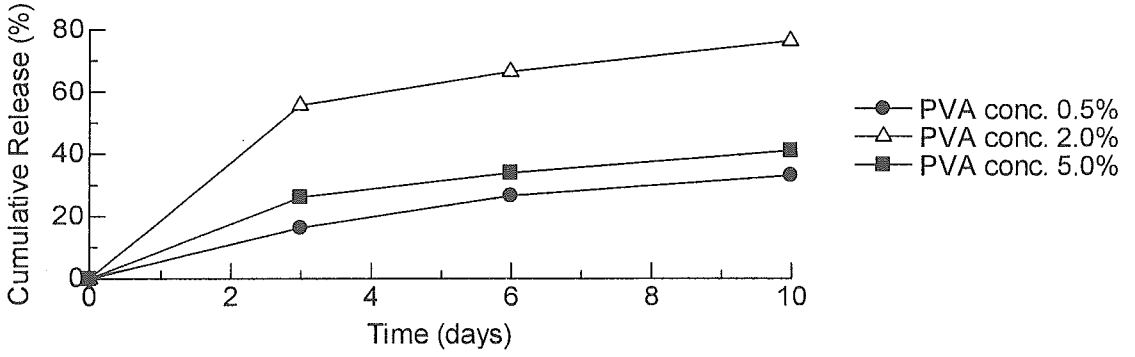

Figure 4. In vitro cumulative BSA release from polydepsipeptide microspheres.

In this study, we have successfully synthesized the sequential polydepsipeptide and prepared the polydepsipeptide microspheres. A model protein, BSA has been encapsulated in the microspheres and released under *in vitro* conditions. These results in this study demonstrate that the polydepsipeptide microspheres are applicable to form a controlled drug delivery system. The protein release profile could be controlled by further study.

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Synthesis and Properties of a Thermo-Responsible Polydepsipeptide Containing Hmb (2-Hydroxy-3-Methylbutanoic Acid) Residues

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We have studied a novel elastin model compound, poly(Gly^1-Val^2-Gly^3-Hmb^4-Ala^5-Pro^6) (1), which has hydroxy acid residues, 2-hydroxy-3-methylbutanoic acid (Hmb). A monomeric fragment, Boc-Gly^1-Val^2-Gly^3-Hmb^4-Ala^5-Pro^6-OBzl was successfully synthesized by the combined method of stepwise elongation and segment condensation in solution states. Thermoresponsive properties were analyzed by variable temperature measurements (such as optical density, ^{1}H NMR, and CD spectra) in H_2O.

Keywords: elastin, self-aggregation, depsipeptide, polymer, coacervation, thermoresponsibility, turbidity.

Introduction

Several water soluble polymers, such as poly(acryloyl-Pro-OMe) (2) [1], poly(methacryloyl-Ala-OEt) (3) [2], poly(*N*-isopropylacrylamide) [3], and an elastin model peptide, poly(Val^1-Pro^2-Gly^3-Val^4-Gly^5) [4], have been known to constitute thermo-responsive materials. These materials show a sharp but continuous volume phase transition from a swollen state to a shrunk state with increasing temperature. This phenomenon is also called as self-aggregation and coacervation. The transition temperature is ranging from near 0°C to over 40°C depending on each chemical structure. Our extensive studied have suggested that the phase transition temperature can be designed simply by changes in amino acid residues and protecting groups by the balancing between hydrophobic and hydrophilic groups in the polymer side chains. Similar results have been reported for elastin model peptides [4], poly(*N*-alkylacrylamide) derivatives [5] and amino acid containing polymers [1,2].

Depsipeptides are oligomers and polymers composed of hydroxy acid and amino acids linked by amide and ester bonds. This hybrid compound attracts attention for their conformational [6] and bioabsorbable properties [7]. In this paper, we have described a novel thermoresponding depsipeptide that is composed of a repetitive sequence, -Gly^1-Val^2-Gly^3-Hmb^4-Ala^5-Pro^6- [8].

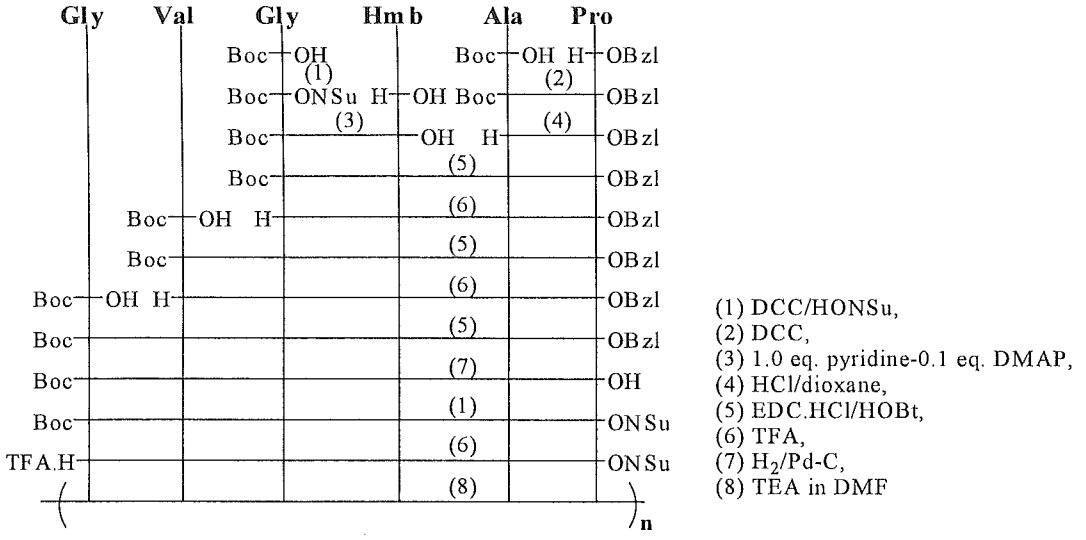

Figure 1. Synthesis of poly(Gly[1]-Val[2]-Gly[3]-Hmb[4]-Ala[5]-Pro[6]) (1).

Results and Discussion

Synthesis of polydepsipeptide

All the compounds used in this study were prepared by the combination of stepwise elongation and segment-condensation methods in solution phase as shown in Fig. 1. We have chosen two protecting groups *tert*-butoxy carbonyl group (Boc-) at the N-terminal and benzyl ester (-OBzl) at the C-terminal. Boc- and -OBzl were removed by acidolysis and hydrogenolysis (Pd-C/H_2), respectively.

A monomeric fragment, Boc-Gly[1]-Val[2]-Gly[3]-Hmb[4]-Ala[5]-Pro[6]-OBzl (**4**) was successfully prepared by the combined method of stepwise elongation and segment condensation reactions in solution states. At first of the synthesis, 2-hydroxy-3-methylbutanoâte pyridinium salt (H-Hmb-OH.pyridine) was coupled with Boc-Gly[3]-ONSu (-ONSu = *N*-hydroxysuccinimide ester) in the presence of 0.1 eq. of dimethylaminopyridine (DMAP) in THF to form Boc-Gly[3]-Hmb[4]-OH, which has an ester connection between Gly[3] and Hmb[4]. The fragment condensation was employed for the synthesis of a four residue fragment, Boc-Gly[3]-Hmb[4]-Ala[5]-Pro[6]-OBzl by the coupling of Boc-Gly[3]-Hmb[4]-OH and HCl.H-Ala[5]-Pro[6]-OBzl by using 1-ethyl-3-(di-methylaminopropyl)-carbodiimide hydrochloride (EDC.HCl) in the presence of an equimolar of 1-hydroxybenzotriazole (HOBt) to suppress racemization and for an efficient reaction.

The hexadepsipeptide free acid, Boc-Gly[1]-Val[2]-Gly[3]-Hmb[4]-Ala[5]-Pro[6]-OH obtained by hydrogenolysis of **4** was derived to an active -ONSu ester by the reaction with HOSu and DCC. Boc-Gly[1]-Val[2]-Gly[3]-Hmb[4]-Ala[5]-Pro[6]-ONSu (**5**) was obtained in 80% yield. The Boc monomer, **5** was treated with 4*M* HCl in dioxane to give a monomer hydrochloride, which were polymerized in dimethylsulfoxide by the addition of triethyl amine. The sequential polydepsipeptide **1** was purified by dialysis in H_2O and obtained higher molecular fraction (m.w. > 3500) was lyophilized. Colorless powder: yield 11% (calculated from **5**), mp. 172-180°C.

Thermal properties of polydepsipeptide

Fig. 2 shows the temperature profiles for turbidity formation of **1** in H_2O and phosphate buffer saline (PBS). As the temperature is raised, the polydepsipeptide, **1**

undergo a phase transition of aggregation in an optical cell. The onset temperature was observed at around 35-40°C in PBS and 45-50°C in H_2O. Therefore, in a physiological environment, such as in PBS, the transition occurs at lower temperature.

Broaden profile was observed in turbidity measurement. In the case of water solution, the phase transition starts relatively sharply at 44°C and continues slowly even at 55°C. Therefore a distinct transition temperature can not be defined in these cases. One explanation for the broaden behavior is due to the molecular weight distribution of **1**. Actually, higher transition temperature was found for lower molecular weight compound.

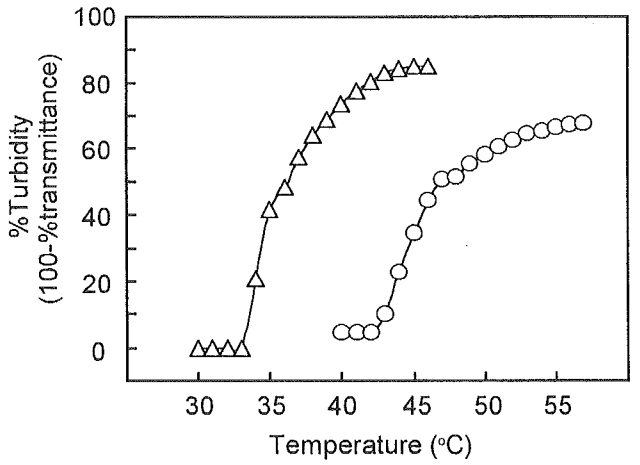

Figure 2. Turbidity measurement of poly(Gly[1]-Val[2]-Gly[3]-Hmb[4]-Ala[5]-Pro[6]) (1) in phosphate buffer saline (△) and in H_2O (○) (1 mg/50 μl, cell length=1 mm). Optical density (% transmittance) was measured at 500 nm.

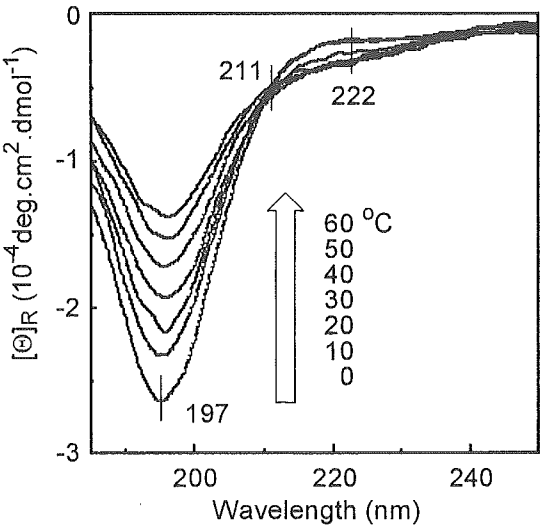

Figure 3. Variable temperature CD spectra of Poly(Gly[1]-Val[2]-Gly[3]-Hmb[4]-Ala[5]-Pro[6]) (1) (in H_2O, 0.1 mg/ml, cell length=1 mm).