ในปีที่สองของโครงการวิจัย ได้ทำการทดสอบความสามารถของ X-ray diffraction quality ของผลึกที่ได้ ณ Synchrotron Radiation Source, Daresbury Laboratory, ประเทศอังกฤษ พบว่า ผลึก PvDHFR-TS/NADPH/pyrimethamine ที่เตรียมได้ diffract synchrotron X-ray radiation ได้เพียงประมาณ 8 Å resolution เท่านั้น ซึ่งไม่เพียงพอในการหาโครงสร้าง แต่นับว่าเป็นความสำเร็จในระดับหนึ่งในการที่เตรียมผลึกได้และทำ preliminary X-ray crystallographic analysis ของผลึกที่ได้ เหตุผลประการหนึ่งที่เป็นไปได้ เนื่องจากผลึกจะต้องผ่านการเดินทางโดยเครื่องบินเป็นเวลามากกว่า 20 ชั่วโมงในสภาวะที่ไม่สามารถควบคุมได้ทั้งอุณหภูมิ ความชื้น ความสั่นสะเทือน และอื่นๆ

แผนการดำเนินการวิจัยในช่วงต่อมาเพื่อปรับปรุงคุณภาพของผลึกที่ได้ และเพื่อทำให้ความสามารถในการกระเจิงรังสีเอกซ์ได้ดีขึ้นกว่าเดิม โดยการหาสภาวะใหม่ในการตกผลึก เติม additive ชนิดต่างๆ เช่น salts, glycerol, detergents ใช้วิธีในการตกผลึกต่างๆ กัน เช่น วิธี hanging drop ควบคู่ไปกับการใช้ microbatch (crystallization under oil) ได้ทำการศึกษาปรับปรุงคุณภาพของผลึกที่ได้โดยเครื่อง R-Axis IV++/rotating-anode x-ray generator ที่ศูนย์วิจัยเพื่อความเป็นเลิศด้านโครงสร้างและหน้าที่การทำงานของโปรตีน (Center for Excellence in Protein Structure and Function) แต่ยังไม่ประสบความสำเร็จในการที่จะได้ผลึกของ bifunctional pvDHFR-TS ที่มีคุณสมบัติดีขึ้นได้มากกว่าเดิม แม้ว่าจะมีการศึกษามากกกว่า 100 ตัวอย่าง

เนื่องจากความพยายามในการปรับปรุงคุณภาพผลึกของ bifunctional pvDHFR-TS ได้ผลไม่เป็นที่น่าพอใจนัก แม้ว่าจะได้ใช้ความพยายาม และเวลาไปอย่างมาก จึงได้เริ่มกลับมาศึกษาความเป็นไปได้ในการตกผลึก genetically engineered DHFR domain ของ pvDHFR-TS แทน ซึ่งพบว่าได้ผลเป็นที่น่าพอใจมากกว่า

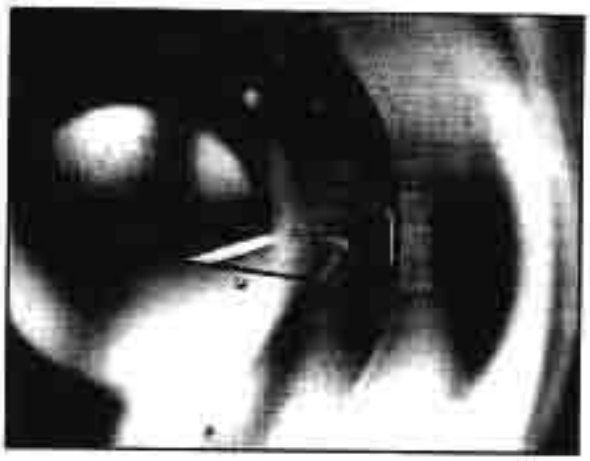
Plasmodium vivax Bifuncational Dihydrofolate reductase (Pv*DHFR*)

Dihydrofolate reductase (DHFR) domain ของ bifuncational DHFR-thymidylate synthase (TS) enzyme ถูก cloned และ overexpressed ใน *E. coli* BL21 pLys[33] และทำเอนไซม์ให้บริสุทธิ์โดยใช้ MTX-affinity column และชะเอา DHFR ออกมาด้วย buffer ที่มี 4 mM DHF ส่วนหนึ่งของเอนไซม์ DHFR ถูกผลิตในรูปของ inclusion body และส่วนที่นำมาทำให้บริสุทธิ์เป็นส่วนของโปรตีนที่ละลายได้ PvDHFR สามารถเตรียมได้ที่ความบริสุทธิ์สูงกว่า 90% และสามารถเตรียมได้ที่ความเข้มข้นประมาณ 4-5 mg/mL และทำการปรับสภาวะของสารละลายโปรตีนให้มีความเข้มข้นของ DHF, salt, buffer ต่ำลง

ผลการวิจัยในระยะแรกพบว่าความสามารถในการละลายของเอนไซม์ PvDHFR ไม่ดี (ละลายได้สูงสุดประมาณ 1 mg/ml ก่อนที่จะตกตะกอน) เมื่อเทียบกับเอนไซม์ PvDHFR-TS (ความเข้มข้นเกินกว่า 10 mg/ml) จากการศึกษาส่วนอื่นเชื่อว่า active site ของ PvDHFR ค่อนข้าง hydrophobic และการเติม inhibitor เข้าไปในสารละลายโปรตีนอาจจะช่วยการละลายของโปรตีนดีขึ้น และทำให้โครงสร้างมีความยืดหยุ่นน้อยลง (less flexible) ซึ่งอาจจะช่วยให้สามารถเตรียมเอนไซม์ที่

ความเข้มข้นสูงขึ้นได้ พบว่าเมื่อเติม pyrimethamine เพื่อไป complex กับ PvDHFR และปรับค่า pH ของสารละลายให้สูงขึ้นจาก pH 6.0 ให้เป็น pH 7.5 ทำให้ความเข้มข้นของโปรตีนที่เตรียมได้มีค่าสูงขึ้นเกือบ 10 mg/ml และเมื่อเติม glycerol และ NaCl ลงในสารละลายด้วยทำให้ความเข้มข้นของเอนไซม์อาจเตรียมได้เกินกว่า 20 mg/ml โดยที่ไม่มีการตกตะกอนเกิดขึ้น การที่สามารถแก้ไขปรับปรุงความสามารถในการละลายของเอนไซม์ pvDHFR ได้ นับว่าเป็นความสำเร็จก้าวสำคัญในการที่จะนำเอนไซม์ที่เตรียมได้ไปศึกษาหาสภาวะที่เหมาะสมในการตกผลึกต่อไป

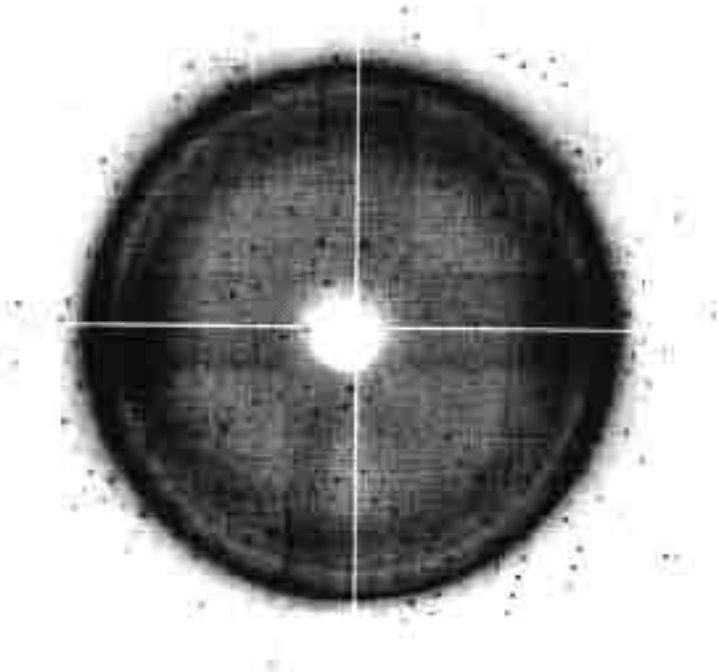
ในการหาสภาวะที่เหมาะสมในการตกผลึกเอนไซม์ pvDHFR นั้น นำสารละลายเอนไซม์ PvDHFR ที่ความเข้มข้นประมาณ 10 mg/ml มาผสมกับ 10 mM NADPH และ 3 mM pyrimethamine (K_i = 0.16 nM) โดยได้เก็บไว้ที่ 4 °C ประมาณ 10-12 ชั่วโมง เพื่อให้เกิด complex อย่างสมบูรณ์ก่อนนำมาทำการทดลอง ในการหาสภาวะในการตกผลึกเบื้องต้น (initial crystallization condition) ใช้ sparse matrix screening conditions[13] ซึ่งเป็นวิธีการสุ่มอย่างกว้างๆ ด้วยสภาวะที่ใช้ได้ผลมาก่อนหน้านี้ (ดู appendix) ด้วยวิธี hanging drop และ microbatch method[37] (crystallization under oil) โดยใช้สารละลาย pvDHFR-NADPH-pyrimethamine 1 – 1.5 μL. รวมกับ crystallizing solution ปริมาณเท่ากัน ขั้นตอนหา crystallization condition นี้ รวมกับการปรับปรุงคุณภาพผลึกที่ได้ใช้เวลาหลายเดือน และพบว่าผลึกที่ดีมีรูปร่างเป็นแบบแผ่น (plate-like) สามารถเตรียมได้จาก 30% polyethylene glycol MW 4000 ที่ pH ประมาณ 6.0-6.5 และ 200 mM ammonium sulfate ที่อุณหภูมิ 4 °C ในสภาวะที่เหมาะสม ผลึกจะเริ่มโตที่เห็นได้ภายใต้กล้องจุลทรรศน์หลังจากวันที่ 3 และโตต่อเนื่องไปถึงประมาณ 2-3 อาทิตย์ โดยมีขนาดเต็มที่ประมาณ 300 × 200 × 150 micron ดังรูปที่แสดง



ผลึกชนิดแผ่นของ wild type PvDHFR-NADPH-pyrimethamine ที่โตเต็มที่ อายุประมาณ 2-3 อาทิตย์

ผลึกของ ternary complex PvDHFR-NADPH-pyrimethamine ได้ถูกนำไปวิเคราะห์เป็นครั้งแรก ที่ Synchrotron Radiation Source, Daresbury Laboratory ประเทศอังกฤษ โดยใช้ beamline 14.2. (ขณะนั้นยังไม่มีการติดตั้ง X-ray detector ที่ Center for Protein Structure and Function หรือที่ภาควิชาเคมี) ผลึกขนาด 200 × 200 × 100 micron ได้ถูกนำขึ้นบนเครื่องวัดโดยใช้ nylon loop ที่อุณหภูมิ 100 Kelvin (ประมาณ -180 °C) ด้วยวิธี flash-freezing[38,39] ผลึกที่ได้กระเจิงรังสี

เอกซ์ชนิด monochromatic (λ = 0.9780 Å) ได้ถึง 2.2 Å resolution โดยเก็บข้อมูลบน detector ชนิด CCD (charge-coupled device) ADSC Quantum-4 ใช้ oscillation angle 1.2° ที่ crystal-to-detector distance 180 mm จำนวน 150 ภาพ ด้วยเวลาภาพละ 45 วินาที ข้อมูลที่ได้ถูกนำมาวิเคราะห์โดยโปรแกรม HKL2000 (Denzo/Scalepack)[40] หรือ CrystalClear



X-ray diffraction pattern ของผลึก PvDHFR-NADPH-pyrimethamine

ข้อมูลที่ได้นำมาวิเคราะห์ด้วยโปรแกรม HKL 2000 (Denzo/Scalepack)[41] หรือ Mosflm/CCP4[42] พบว่า ผลึกของ PvDHFR-NADPH-pyrimethamine มีกลุ่มสมมาตรชนิด monoclinic มี space group เป็น C2 มี unit cell parameter คือ a = 136.53 Å, b = 55.61 Å, c = 45.75 Å and β = 107.46° ข้อมูลที่ได้มีความละเอียดถึง 2.4 Å resolution มีค่า mosaicity ประมาณ 0.6 ° มีความสมบูรณ์สูงถึง 99.8% ค่า $I/\sigma(I)$ 19.5 ซึ่งนับว่ามี intensity ที่ดี มีค่า R_{merge} 6% (11.5% สำหรับ resolution 2.40-2.49 Å) unique reflection จำนวน 16,385 ขนาดของ unit cell ที่ได้สอดคล้องกับ DHFR (27 kDa) จำนวน 1 โมเลกุลในแต่ละ asymmetric unit มี solvent content ประมาณ 58% (Matthews' constant 3.0)[14]

Data collection statistics of WtPvDHFR-NADPH-Pyr

```
Crystal parameters:

 Unit cell lengths:  134.2848   55.7365   45.7163
 Unit cell  angles:   90.0000  107.0851   90.0000
 Unit cell  volume: 327066.605
Orientation angles:   24.1589  -59.3422  -86.6694
        Mosaicity:     0.620

Spacegroup number: Sname: C2
```

Summary of reflections intensities and R-factors by shells

Shell limit	Lower Angstrom	Upper	Average I	Average error	Average stat.	Norm. Chi**2	Linear R-fac	Square R-fac
33.00	5.16		2635.9	164.4	87.7	0.756	0.059	0.131
5.16	4.10		2817.7	119.5	67.0	0.863	0.036	0.073
4.10	3.58		1799.3	77.1	43.3	0.995	0.036	0.042
3.58	3.26		953.8	43.0	26.4	1.008	0.041	0.045
3.26	3.02		485.1	20.0	16.4	1.000	0.043	0.045
3.02	2.85		251.5	13.5	12.2	0.972	0.060	0.067
2.85	2.70		169.3	11.7	11.1	0.826	0.071	0.075
2.70	2.59		111.3	11.2	10.9	0.830	0.098	0.113
2.59	2.49		99.2	13.3	13.1	0.863	0.105	0.113
2.49	**2.40**		**81.2**	**13.5**	**13.1**	**0.657**	**0.115**	**0.121**
All reflections			**955.9**	**49.0**	**30.2**	**0.891**	**0.046**	**0.096**

อนึ่ง หลังจากการติดตั้ง X-ray detector ที่ Center for Protein Structure and Function ผลึกที่ทำการเก็บข้อมูลได้ดีถึงประมาณ 1.8 Å resolution การหาโครงสร้างผลึกของ ternary complex PvDHFR-NADPH-pyrimethamine ใช้วิธี molecular replacement ด้วยโปรแกรม AMoRe[17] ใน CCP4 program suite[43] และโปรแกรม CNS[16] โดยใช้ incomplete coordinates ของ *Plasmodium falciparum* DHFR-TS เป็นจุดเริ่มต้น โดยมีผลการหา rotation function search และ translation function search ดังตาราง

Rotation Function Searches

```
! total number of reflections used:          3223 (  81.3 % )
! mean of rotation function:                0.0530
! standard deviation around mean:           0.0264
 ! index,  theta1,  theta2,  theta3,  RF-function (EPSIlon= 0.25)
      1   259.545   25.670  195.125     0.2312 (8.8 s)
      5   249.441   31.443  211.003     0.1026 (3.8 s)
      6   266.264   64.330  190.982     0.0952
      8   254.196   19.896  189.776     0.0922
      9   275.580   19.896  185.713     0.0899
     10   246.874   47.009  123.114     0.0888
     11   253.771   22.783  215.333     0.0875
     12   269.003   19.330  161.994     0.0868
     14   260.743   61.443  180.264     0.0842
     15   255.643   70.104  137.079     0.0842
     18   244.025   57.991  136.025     0.0828
     19   229.863   -1.443  242.854     0.0818
     21    90.911   73.557  195.617     0.0810
```

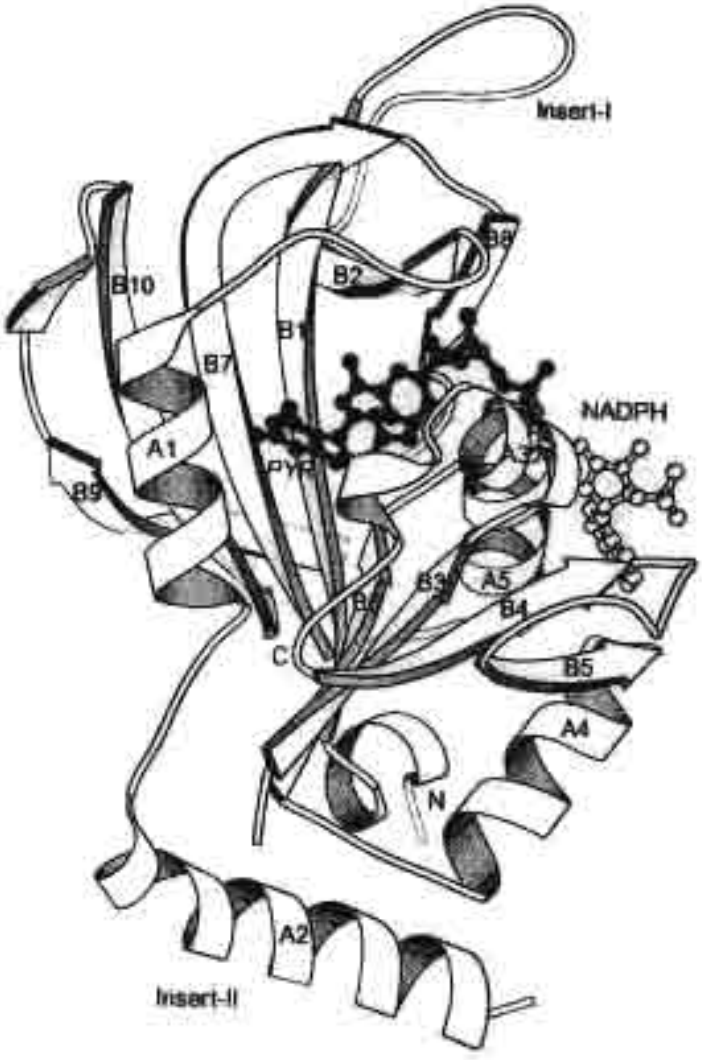
Translation Function Searches

		theta1	theta2	theta3	transX	transY	transZ	monitor	packing
R#	1	**259.30**	**26.87**	**195.14**	**18.28**	**0.82**	**16.17**	**0.447**	**0.4866**
R#	2	259.32	26.83	195.11	-46.99	0.53	16.13	0.447	0.4861
R#	3	268.18	63.36	190.50	7.44	-0.02	13.03	0.202	0.3681
R#	4	259.36	26.86	195.09	18.26	4.23	16.16	0.447	0.4866
R#	5	273.74	19.44	185.10	17.00	0.40	12.35	0.196	0.4887
R#	6	248.34	46.79	120.66	-11.32	-1.85	4.73	0.245	0.4170
R#	7	259.32	26.86	195.12	18.27	7.77	16.16	0.447	0.4864
R#	8	274.18	16.40	154.58	16.18	-3.06	12.74	0.216	0.4351
R#	9	263.40	58.90	179.61	24.60	-0.14	11.35	0.200	0.4819
R#	10	255.20	67.79	138.99	14.32	1.58	12.16	0.195	0.3778

ผลจากการหาโครงสร้างด้วยเทคนิค molecular replacement ได้ผลที่ดีที่สุดคือ

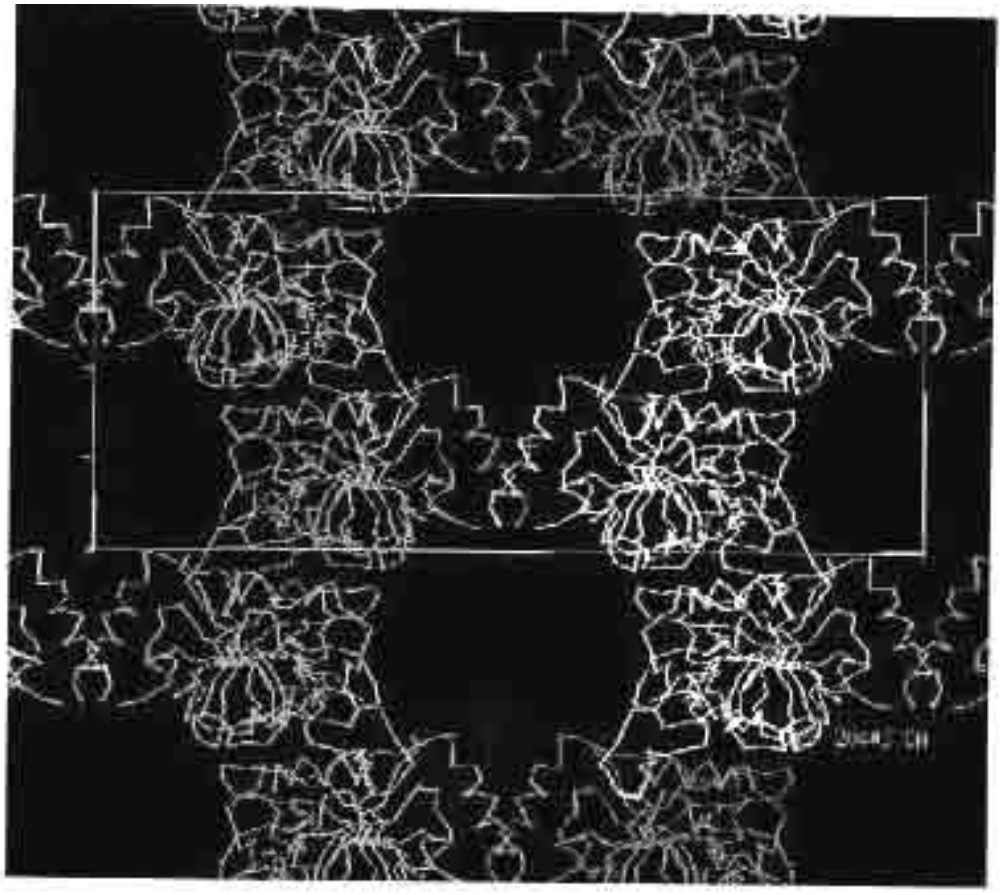
	theta1	theta2	theta3	transX	transY	transZ	monitor	packingR#
7	259.32	26.86	195.12	18.27	7.77	16.16	0.447	0.4864

เมื่อนำค่าที่ได้ไปคำนวณต่อ และวิเคราะห์คุณภาพของ electron density map โดยใช้ molecular graphics program XtalView[18,19] พบว่ามีความต่อเนื่องและมีความสอดคล้องกับโครงสร้างในระดับหนึ่ง โดยมีบางบริเวณของโครงสร้างไม่มี electron density เนื่องจากมีโครงสร้างที่ต่างกัน รวมทั้งในบริเวณของ side chain และ loop บางส่วนด้วย โครงสร้างที่ได้ได้ผ่านการแก้ไข manual model building และ editing และกระบวนการ refinement เพื่อลดข้อผิดพลาด หลายรอบจนกระทั่งได้โครงสร้างที่เกือบสมบูรณ์โดยมีบริเวณ residue ที่ 85-105 ที่ไม่พบใน electron density map เนื่องจากการ disorder



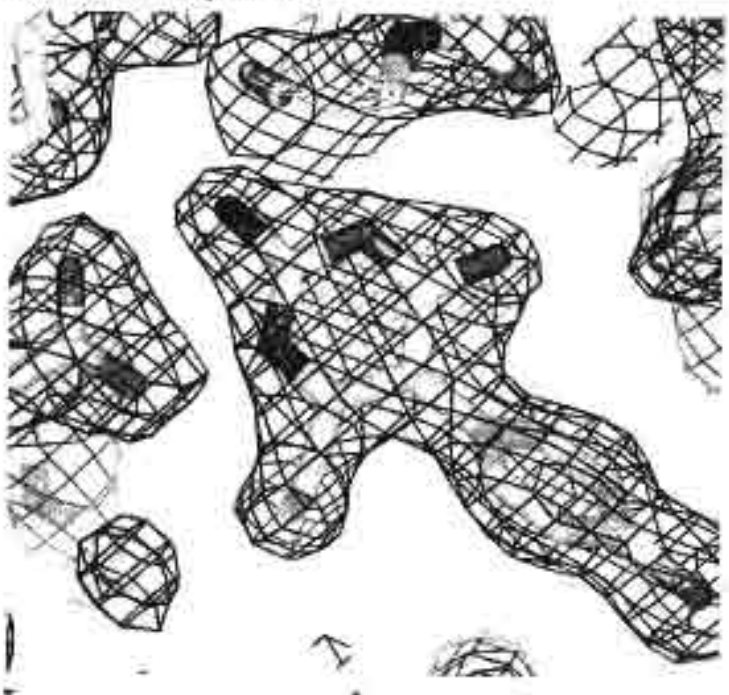
โครงสร้างของ *P. vivax* DHFR-NADPH-pyrimethamine ในแบบ ribbon diagram โมเลกุลของ NADPH และ pyrimethamine แสดงด้วย ball-and-stick model

โครงสร้างของ *P. vivax* DHFR เป็นชนิด α/β ที่มี 8-β-stranded sheet อยู่ตรงกลางและมี α-helix อยู่ด้านนอก ส่วนของ insert-1 ที่อยู่ระหว่าง B-1 และ B-2 เป็น loop ที่ไม่มี secondary structure ส่วนของ insert-2 มีโครงสร้างเป็น helix และมีบางส่วนที่ disorder ซึ่งทั้งสองบริเวณนี้เป็นส่วนที่พิเศษของเอนไซม์ DHFR ในกลุ่ม *Plasmodium* ทั้งสองส่วนนี้เป็นบริเวณที่มี flexibility สูง เมื่อเทียบกับบริเวณอื่นๆ สันนิษฐานได้ว่าอาจเป็นบริเวณที่เกี่ยวข้องกับ interaction กับ partner molecule อื่นๆ เนื่องจากส่วนเพิ่มเติมทั้งสองส่วนนี้ ห่างจาก binding site จึงไม่น่าจะมีส่วนเกี่ยวข้องในการเกิดปฏิกิริยาหรือการจับกับยาของเอนไซม์นี้

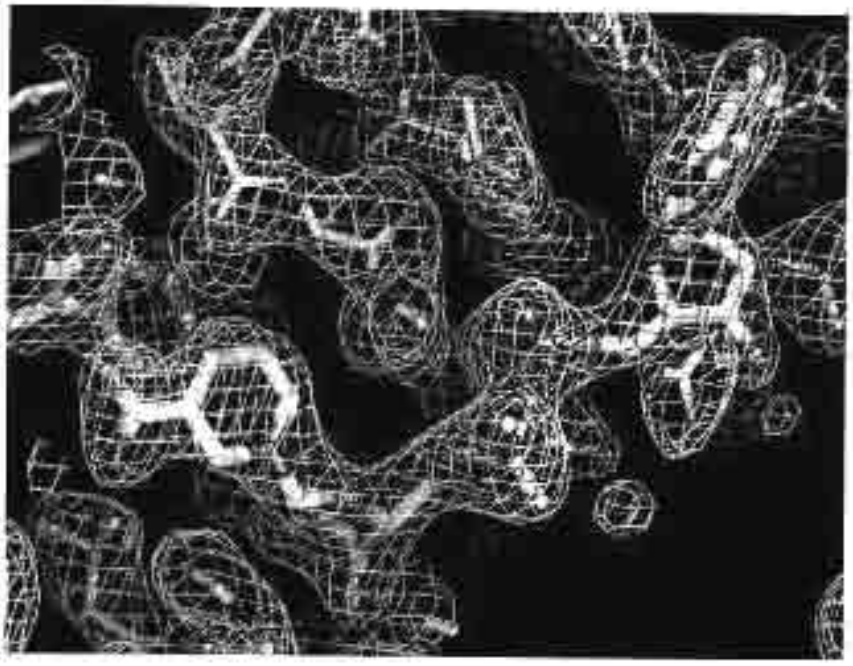


Packing diagram ของ *P. vivax* DHFR-NADPH-pyrimethamine ใน monoclinic C2 โดยมี 1 molecule ของ DHFR ในแต่ละ asymmetric unit

ในบริเวณ antifolate binding site สามารถเห็นโมเลกุลของ pyrimethamine และ NADPH จับอยู่กับเอนไซม์ได้อย่างชัดเจนดังรูปที่แสดง

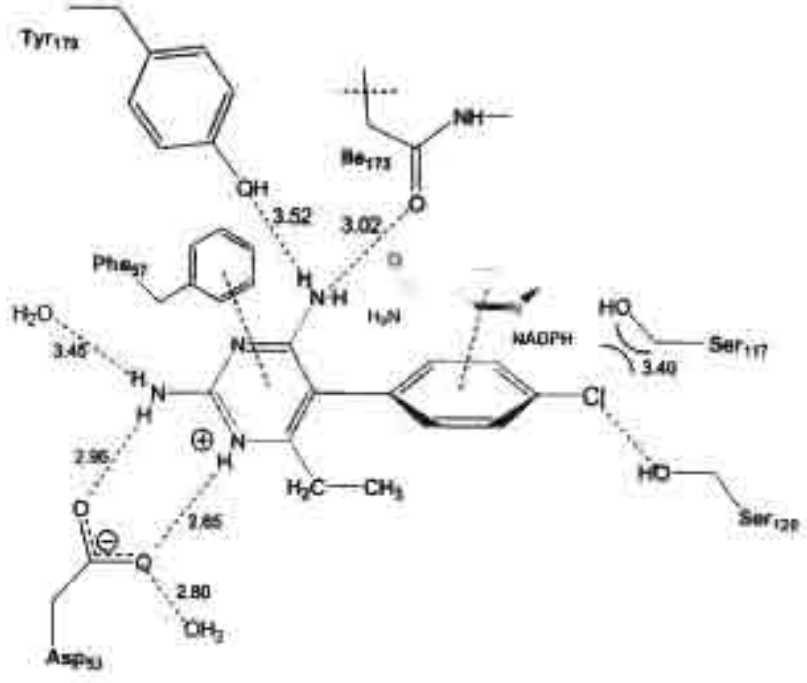


โมเลกุลของ pyrimethamine ใน binding site ของ *P. vivax* DHFR

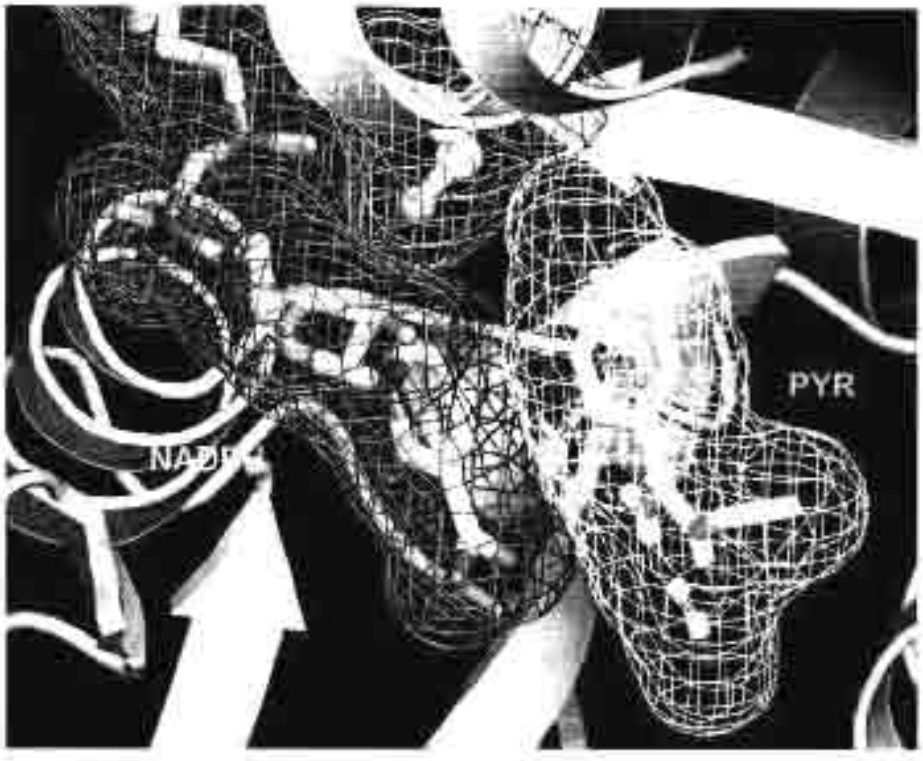


โมเลกุลของ NADPH ใน binding site ของ *P. vivax* DHFR

เมื่อพิจารณา inhibitor binding site ของ *P. vivax* DHFR เพื่อทำความเข้าใจถึงกลไกการทำงานของ inhibitor ในกลุ่ม antifolate พบว่า ส่วนของวง pyrimidine จับตัวผ่าน strong hydrogen bond กับ carboxylate group ของ aspartate-53 โดยมีระยะทางระหว่าง heteroatom (N-O) ประมาณ 2.5 และ 2.8 Å กลุ่ม amino ที่ C-4 เกิดพันธะ hydrogen ที่อ่อนกว่ากับ main chain oxygen ของ isoleucine-173 และกับกลุ่ม hydroxyl side chain ใน tyrosine-179 Phe-179 ที่ conserved ใน DHFR เกิด π–π interaction กับกลุ่ม pyrimidine โดยมีระยะห่างระหว่าง plane ประมาณ 3.7 Å Interaction กับ cofactor NADPH เป็นส่วนสำคัญส่วนหนึ่งในการจับตัวกันของ antifolate



A schematic diagram แสดง pyrimethamine binding site ใน *P. vivax* DHFR (distance shown in Å unit.)



Interaction between pyrimethamine to NADPH and Ser117. Meshed surface drawn according to the covalent radii of the molecules.

Antifolate resistance mechanism

จากข้อมูลทางระบาดวิทยาพบว่าการเกิดการดื้อยาของเชื้อมาลาเรียชนิด *P. vivax* ส่วนใหญ่เกิดขึ้นที่ codon ของ Ser117[33-35,44,45] ที่เกิดการกลายเป็น Asn จาก diagram แสดง antifolate binding site ใน *P. vivax* DHFR พบว่า phenyl ring ของ pyrimethamine เกิด π–π interaction กับกลุ่ม nicotinamide ของ NADPH (ประมาณ 1-5 kcal/mol) กลุ่ม chlorine ที่ตำแหน่ง para- ของ phenyl ring นั้นอยู่ใกล้กับ Ser117 โดยมีระยะห่างประมาณ 3.4 Å แต่ภาพที่แสดงข้างต้นแสดงให้เห็นคุณสมบัติที่ชัดเจนคือการที่มี van der Waal's interaction กับเอนไซม์ด้วย การที่ mutation ที่ตำแหน่ง 117 จาก serine ในเอนไซม์ชนิด wild type เป็น asparagine ที่มีขนาดใหญ่ขึ้นจะทำให้การจับของ pyrimethamine กับเอนไซม์ไม่ดีดังเดิม โดยเกิด steric interaction กับกลุ่ม para-chloro และอาจเป็นผลให้สูญเสียทั้ง π–π interaction และ/หรือ van der Waal's interaction ไปบางส่วน ผลของการเกิด mutation นี้ทำให้เกิดการดื้อยา pyrimethamine กว่า 300 เท่า ($K_{i(wild\ type)}$ = 0.16 nm, $K_{i(S117N,S58R)}$ = 50 nm) การพิสูจน์กลไกการดื้อยานี้ ต้องมีการศึกษาเพิ่มเติมต่อไปโดยการศึกษาโครงสร้างของยาในการจับตัวกับเอนไซม์ที่เกิดการกลายพันธุ์ (mutant enzyme) ต่อไป

ผลงานวิจัยเกี่ยวกับการศึกษาการดกผลึกของเอนไซม์ *P. vivax* DHFR ได้เตรียมต้นฉบับเพื่อส่งตีพิมพ์ในวารสาร *Acta Crystallographica* Section D และผลงานวิจัยเกี่ยวกับการศึกษาโครงสร้างของ *P. vivax* DHFR-NADPH-pyrimethamine กำลังเตรียมต้นฉบับเพื่อส่งพิจารณาตีพิมพ์ในวารสาร *Science*

งานวิจัยในส่วนของการศึกษาโครงสร้างสามมิติของ *P. vivax* DHFR เป็นการร่วมมือกับ ดร. อุบลศรี เลิศสกุลพานิช และ ศ.ดร.ยงยุทธ ยุทธวงศ์ ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ

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รายชื่อนักศึกษาที่เกี่ยวข้อง

นักศึกษาระดับปริญญาโทที่จบการศึกษา

Mr. Man Theerasilp: M.Sc. Physical Chemistry, graduated April 2003. The Reaction between artemisinin and ferroprotoporphyrin IX: Stoichiometric and kinetic studies.

นักศึกษาระดับปริญญาเอก

Miss Chariwat Samanchart, M.Sc. study, Organic Chemistry, Mahidol University, Research area: X-ray crystallographic analysis of *Pseudomonas stutzeri* D-phenylglycine aminotransferase

นักศึกษาระดับปริญญาโท

Mr. Puttapol Khongsuk, M.Sc. study, Chemistry, Mahidol University, Research area: X-ray crystallographic analysis of *Plasmodium vivax* dihydrofolate reductase-thymidylate synthase

Miss Anothai Supanpong, M.Sc. study, Organic Chemistry, Mahidol University, Research area: *Plasmodium vivax* dihydrofolate reductase-thymidylate synthase

Miss Kornkamon Kalapakornchai, M.Sc. study, Organic Chemistry, Mahidol University, Research area: Enantioselective synthesis of β-glucosides using Cassava linamarase as a biocatalyst.

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Miss Supreeya Cheewarat, M.Sc. study, Organic Chemistry, Mahidol University, Research area: Bioactive compounds from microorganisms

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Miss Srisuda Trirakarn, B. Sc. Study, Chemistry, Mahidol University.
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Miss Tippawan Katekaew, B. Sc. Study, Chemistry, Mahidol University.
Research area: Enantioselective synthesis of β-glucosides using Cassava linamarase as a biocatalyst.

Miss Aimon Thongpanchang, B. Sc. Study, Chemistry, Mahidol University.
Research area: Metacycloprodigiosin: its DNA-binding mechanism.

กิจกรรมอื่น ๆ ที่เกี่ยวข้อง

Oral Presentation

1. X-ray crystallographic structure setermination of D-phenylglycine aminotransferase. (invited speaker) Protein Research Network Symposium 2002. Mahidol University, Bangkok, August 29-30, 2002.

2. X-Ray crystal structure of dihydrofolate reductase from *Plasmodium vivax*: antifolate-resistance mechanism. (invited speaker) Postgraduate Education and Research in Chemistry Conference II Jomtien Palm Beach Hotel, Pattaya, Thailand,11-14 May, 2003.

3.การศึกษาโครงสร้างสามมิติด้วยรังสีเอกซ์ในงานวิจัยทางเคมีและชีววิทยา
Structural X-ray Crystallographic Investigation in Chemistry and Biology Research: from bioactive compounds to cellular targets. (invited speaker) การประชุมวิชาการวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย (วทท.) ครั้งที่ 29 วันที่ 20-22 ตุลาคม 2546 ณ ศูนย์ประชุมอเนกประสงค์กาญจนาภิเษก มหาวิทยาลัยขอนแก่น

4. X-ray Crystallography in a Modern Era: a journey of small to large molecules" Chemistry Colloquium ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล 5 พฤศจิกายน 2546

5. X-ray crystal structures of *Plasmodium vivax* dihydrofolate reductase: a clue to antifolate drug resistance. คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย 12 พฤศจิกายน 2546

รางวัลที่ได้รับเกี่ยวกับงานวิจัย

รางวัลนักวิทยาศาสตร์รุ่นใหม่ ประจำปี 2546 จากมูลนิธิส่งเสริมวิทยาศาสตร์และเทคโนโลยี ในพระบรมราชูปถัมภ์ วันที่ 18 สิงหาคม 2546

ตำแหน่งวิชาการ

ดร.พลังพล คงเสรี ได้รับการเลื่อนตำแหน่งวิชาการเป็นรองศาสตราจารย์ ในเดือนกรกฎาคม 2546

ข้อมูลอื่น ๆ

คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ได้สนับสนุนให้จัดตั้งศูนย์วิจัยเพื่อความเป็นเลิศด้านโครงสร้างและหน้าที่การทำงานของโปรตีน (Center for Excellence in Protein Structure and Function) ที่ชั้น 4 อาคารเฉลิมพระเกียรติ เพื่องานวิจัยด้าน structure biology และ enzyme mechanism and function โดยมี ศ. ดร. ม.ร.ว. ชิษณุสรร สวัสดิวัตน์ เป็นหัวหน้าศูนย์ และ กลุ่มวิจัยของดร.พลังพล คงเสรี ได้เป็นนักวิจัยในหน่วยวิจัยนี้ โดยคณะวิทยาศาสตร์ ได้จัดสรรงบประมาณเพื่อสนับสนุนในการจัดหาอุปกรณ์วิทยาศาสตร์ ขนาดใหญ่ เช่น R-AXIS IV++ X-ray diffractometer และอุปกรณ์ขนาดใหญ่อื่นๆ เงินวิจัยที่ได้รับการสนับสนุนจาก สกว. เป็นส่วนหนึ่งของ counter funding เพื่อใช้ในงานวิจัยเกี่ยวกับ structural biology

Appendix I

Sparse-matrix screening conditions

No.	Precipitant	Buffer and pH	Salt
1.	30%MPD	0.1 M Na acetate pH 4.5	0.02 M $CaCl_2$
2.	0.4 M K / Na tartrate	None	None
3.	0.4 M AP	None	None
4.	2.0 M AS	0.1 M Tris base pH 8.5	None
5.	30%MPD	0.1 M HEPES pH 7.5	0.2 M Na citrate
6.	30%PEG 4000	0.1 M Tris base pH 8.5	0.2 M $MgCl_2$
7.	1.4 M Na acetate	0.1 M MES pH 6.5	None
8.	30%*iso*-Propanol	0.1 M MES pH 6.5	0.2 M Na citrate
9.	30%PEG 4000	0.1 M Na citrate pH 5.5	0.2 M AA
10.	30%PEG 4000	0.1 M Na acetate pH 4.5	0.2 M AA
11.	1.0 M AP	0.1 M Na citrate pH 5.5	None
12.	30%*iso*-Propanol	0.1 M HEPES pH 7.5	0.2 M $MgCl_2$
13.	30%PEG 400	0.1 M Tris base pH 8.5	0.2 M Na citrate
14.	28%PEG 400	0.1 M HEPES pH 7.5	0.2 M $CaCl_2$
15.	30%PEG 8000	0.1 M MES pH 6.5	0.2 M AS
16.	1.5 M Li_2SO_4	0.1 M HEPES pH 7.5	None
17.	30%PEG 4000	0.1 M Tris base pH 8.5	0.2 M Li_2SO_4
18.	20%PEG 8000	0.1 M MES pH 6.5	0.2 M $Mg(CH_3COO)_2$
19.	30%*iso*-Propanol	0.1 M Tris base pH 8.5	0.2 M AA
20.	25%PEG 4000	0.1 M Na acetate pH 4.5	0.2 M AS
21.	30%MPD	0.1 M MES pH 6.5	0.2 M $Mg(CH_3COO)_2$
22.	30%PEG 4000	0.1 M Tris base pH 8.5	0.2 M Na acetate
23.	30%PEG 400	0.1 M Na HEPES pH 7.5	0.2 M $MgCl_2$
24.	20%*iso*-Propanol	0.1 M Na acetate pH 4.5	0.2 M $CaCl_2$

No.	Precipitant	Buffer and pH	Salt
25.	1.0 M Na acetate	0.1 M Imidazole pH 6.5	None
26.	30%MPD	0.1 M Na citrate pH 5.5	0.2 M AA
27.	20%*iso*-Propanol	0.1 M HEPES pH 7.5	0.2 M Na citrate
28.	30%PEG 8000	0.1 M MES pH 6.5	0.2 M Na acetate
29.	0.8 M K/Na tartrate	0.1 M HEPES pH 7.5	None
30.	30% PEG 8000	None	0.2 M AS
31.	30% PEG 4000	None	0.2 M AS
32.	2.0 M AS	None	None
33.	4.0 M Na acetate	None	None
34.	2.0 M Na acetate	0.1 M Na acetate pH 4.5	None
35.	0.8 M NaH_2PO_4 / 0.8 M K_2HPO_4	0.1 M HEPES pH 7.5	None
36.	8%PEG 8000	0.1 M Tris base pH 8.5	None
37.	8%PEG 4000	0.1 M Na acetate pH 4.5	None
38.	1.2 M Na citrate	0.1 M HEPES pH 7.5	None
39.	2%PEG 400	0.1 M HEPES pH 7.5	2.0 M AS
40.	20%*iso*-Propanol / 20%	0.1 M Na citrate pH 5.5	None
41.	10 %*iso*-Propanol / 20%	0.1 M HEPES pH 7.5	None
42.	20%PEG 8000	None	0.05 M K_2HPO_4
43.	30%PEG 1500	None	None
44.	0.2 M Mg acetate	None	None
45.	18%PEG 8000	0.1 M MES pH 6.5	0.2 M $Zn(CH_3COO)_2$
46.	18%PEG 8000	0.1 M MES pH 6.5	0.2 M $CaCl_2$
47.	2.0 M AS	0.1 M Na acetate pH 4.5	None
48.	2.0 M AP	0.1 M Tris base pH 8.5	None

Appendix II

Reprint

M. Isaka, A. Jaturapat, M. Tanticharoen, **P. Kongsaeree**, Y. Thebtaranonth.
Aigialomycins A-E, New Resorcylic Macrolides from
the Marine Mangrove Fungus *Aigialus parvus*.
J. Org. Chem., **2002**, 67 (5), 1561-1566.

Aigialomycins A−E, New Resorcylic Macrolides from the Marine Mangrove Fungus *Aigialus parvus*

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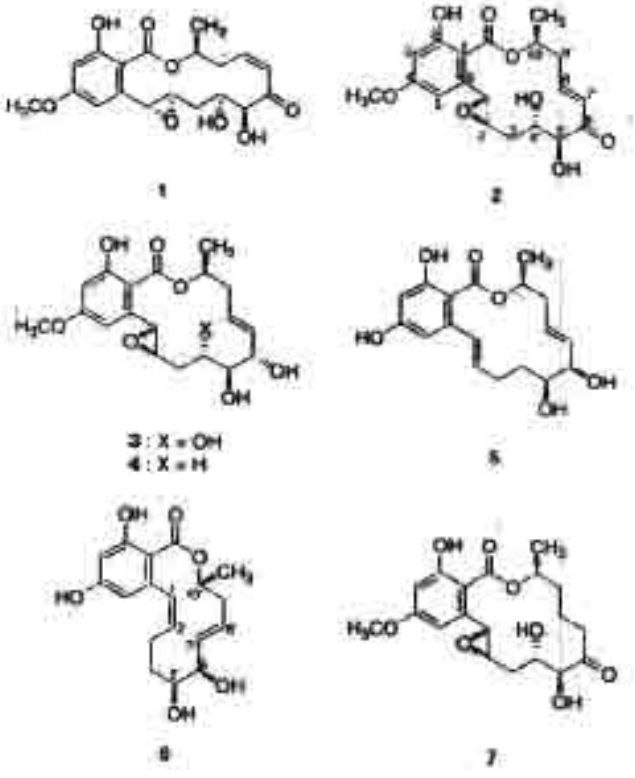
Aigialomycins A−E (**2**−**6**), new 14-membered resorcylic macrolides, were isolated together with a known hypothemycin (**1**) from the mangrove fungus, *Aigialus parvus* BCC 5311. Structures of these compounds, including absolute configuration, were elucidated by spectroscopic methods, chemical conversions, and X-ray crystallographic analysis. Hypothemycin and aigialomycin D (**5**) exhibited in vitro antimalarial activity with IC_{50} values of 2.2 and 6.6 μg/mL, respectively, while other analogues were inactive. Cytotoxicities of these compounds were also evaluated.

Marine microorganisms have proved to be rich sources of bioactive secondary metabolites, and numerous compounds with potent biological activities and unique chemical structures have been isolated.[1] In the course of our search for novel bioactive compounds from microbial sources,[2] we came across antimalarial activity (IC_{50} 8 μg/mL) of an extract from a lignicolous mangrove ascomycete, *Aigialus parvus* BCC 5311. Since there has been no report on the secondary metabolites of the genus *Aigialus*, we decided to undertake the investigation of the antimalarial constituents of the strain BCC 5311. Activity-guided fractionation and chromatographic separation led to the isolation of a known resorcylic macrolide, hypothemycin (**1**),[3] as the major secondary metabolite together with its five new analogues, named aigialomycins A−E (**2**−**6**). We report herein the isolation, structural elucidation, and biological activities of these novel macrolides.



Results and Discussion

The major metabolite of *A. parvus* BCC 5311, compound **1**, was obtained as colorless needles. The structure of this compound was elucidated by spectroscopic method (NMR, MS, IR, and UV). Its melting point (170−172 °C), specific rotation ($[\alpha]^{25}_{D}$ −18; *c* 0.50, $CHCl_3$), and other spectral data were identical to those of hypothemycin whose structure has previously been determined by an X-ray crystallographic analysis.[4]

Aigialomycin A (**2**) was isolated as the second major secondary metabolite. HRMS and elemental analysis indicated that this compound possesses the same molecular formula as hypothemycin ($C_{18}H_{22}O_8$). NMR analyses (^1H, ^{13}C, DEPTs, COSY, HMQC, and HMBC) revealed that the configuration at C-7′−C-8′ double bond of

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(1) (a) Faulkner, D. J. *Nat. Prod. Rep.* **2000**, *17*, 1−6. (b) Faulkner, D. J. *Nat. Prod. Rep.* **2001**, *18*, 1−49.

(2) (a) Isaka, M.; Tanticharoen, M.; Kongsaeree, P.; Thebtaranonth, Y. *J. Org. Chem.* **2001**, *66*, 4803−4808. (b) Isaka, M.; Kongsaeree, P.; Thebtaranonth, Y. *J. Antibiot.* **2001**, *54*, 36−43. (c) Isaka, M.; Tanticharoen, M.; Thebtaranonth, Y. *Tetrahedron Lett.* **2000**, *41*, 1657−1660. (d) Isaka, M.; Punya, J.; Lertwerawat, Y.; Tanticharoen, M.; Thebtaranonth, Y. *J. Nat. Prod.* **1999**, *62*, 329−331. (e) Ekthawatchai, S.; Isaka, M.; Kittakoop, P.; Kongsaeree, P.; Sirichaiwat, C.; Tanticharoen, M.; Tarnchompoo, B.; Thebtaranonth, Y.; Yuthavong, Y. *J. Heterocycl. Chem.* **1999**, *36*, 1599−1605.

(3) (a) Nair, M. S. R.; Carey, S. T. *Tetrahedron Lett.* **1980**, *21*, 2011−2012. (b) Nair, M. S. R.; Carey, S. T.; James, J. C. *Tetrahedron* **1981**, *37*, 2445−2449.

(4) Revised structure of hypothemycin: Agatsuma, T.; Takahashi, A.; Kabuto, C.; Nozoe, S. *Chem. Pharm. Bull.* **1993**, *41*, 373−375.

10.1021/jo010930g CCC: $22.00
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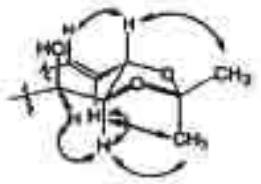
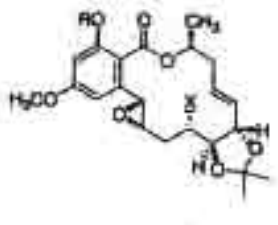


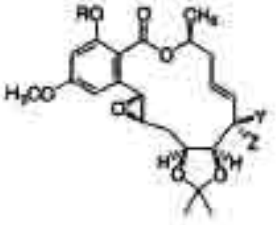
Figure 1. Selected NOESY correlations for **10** (in methanol-d_4).

aigialomycin A (**2**) differed from that of **1**, having the *trans* geometry ($J_{7',8'} = 15.8$ Hz). Catalytic hydrogenation of compounds **1** and **2** (H_2, 10% Pd/C, EtOAc) gave the same 7′,8′-dihydro analogue **7**: $[\alpha]^{26}_D$ −16 (*c* 0.17, $CHCl_3$) and $[\alpha]^{27}_D$ −14 (*c* 0.17, $CHCl_3$), respectively. Therefore, the 1′*R*,2′*R*,4′*S*,5′*S*,10′*S* configuration of aigialomycin A (**2**), identical to **1**, was established. Physicochemical properties (mp, UV, IR, and ^{1}H NMR) of compound **7** were consistent to those of dihydrohypothemycin which was previously isolated, along with hypothemycin, from *Hypomyces trichothecoides*.[3b] Revised ^{1}H NMR assignments and ^{13}C NMR data of **7** are presented in Experimental Section.

The molecular mass ion $[M + H]^+$ of aigialomycin B (**3**) is 2 mass units more than compound **2**, and the molecular formula of $C_{19}H_{24}O_8$ was established by HRMS and NMR. IR and ^{13}C NMR spectra of **3** indicated the absence of ketone functionality. NMR analyses, particularly COSY, HMQC, and HMBC, clearly revealed that compound **3** was an analogue of **2** but with a different oxidation state at C-6′. The relative configuration of the triol moiety (C-4′ to C-6′) was determined by chemical means. Thus, treatment of **3** under the acetonide-forming conditions (2,2-dimethoxypropane, cat. TsOH, rt, 2 h) resulted in an inseparable mixture of two acetonide isomers (**8** and **9**, analyzed by NMR data) which upon methylation (MeI/K_2CO_3 in 2-butanone, rt, 20 h) followed by preparative reversed-phase HPLC separation provided compounds **10** and **11** (**10**:**11** = 65:35; 95% yield). The $J_{5',6'}$ value of 8.0 Hz (in methanol-d_4) suggested a *cis*-fused acetonide structure for **10**; however, the NOESY spectrum indicated otherwise. Correlation between H-5′ and the δ_H 1.42 acetonide methyl was evident while H-6′ correlated to the δ_H 1.45 acetonide methyl. Also, the intensity of NOESY cross signal between H-5′ and H-6′ was weak, and therefore, H-5′ and H-6′ should be placed on the opposite side of the acetonide ring (*trans*-fused acetonide). The relative configuration from C-4′ to C-8′ in **10** is shown in Figure 1. Compound **11**, $J_{4',5'} = 5.0$ Hz, was assigned as *cis*-fused acetonide by the observation of NOESY correlations (in $CDCl_3$) from both H-4′ and H-5′ to the same acetonide methyl at δ_H 1.37, and not to the other at δ_H 1.49. These experimental results revealed that two hydroxyl groups on C-4′ and C-5′ of aigialomycin B (**3**) are attached to the same side of the macrolide ring and are opposite to that of the hydroxyl group on C-6′. $NaBH_4$ reduction (THF/MeOH, 0 °C, 20 min) of compound **12**, acetonide of aigialomycin A, followed by methylation (MeI, K_2CO_3, 2-butanone) and purification by prep HPLC provided a mixture of two products in a ratio 2:1. Attempted separation of this mixture failed. However, NMR analysis of the mixture revealed that the major product was identical to compound **11** obtained previously from the reaction described above. The minor product, presumably C-6′ epimer of **11**, was not identified. Thus, aigialomycin B (**3**) has a relative configuration identical to **2** and on the basis of these chemical conversions, the 1′*R*,2′*R*,4′*S*,5′*S*,6′*S*,10′*S* configuration, as depicted, was determined for **3**.



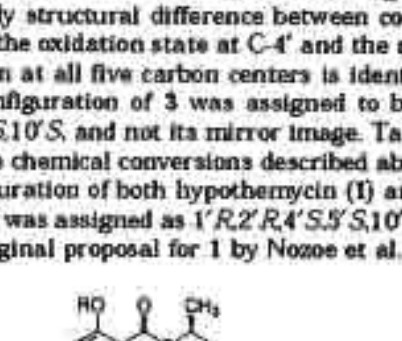
8: R = H; X = OH
10: R = CH_3; X = OH
13: R = CH_3; X = H



9: R = H; Y = H, Z = OH
11: R = CH_3; Y = H, Z = OH
12: R = H; Y + Z = O

Aigialomycin C (**4**), having molecular formula of $C_{19}H_{24}O_7$ (HRMS, NMR), exhibited a structure similar to **3** as elucidated by NMR analyses. The derivative **13**, prepared in two steps from **4**, was found to be a *trans*-fused acetonide by the analysis of NOESY spectra from which the same correlation information as compound **10** was obtained. Thus, aigialomycin C (**4**) was assigned as the 4′-dehydroxy analogue of aigialomycin B (**3**).

There remains an uncertainty on the previously proposed absolute configuration of 1′*R*,2′*R*,4′*S*,5′*S*,10′*S* for **1**, since the elucidation was based on the scattering method using anomalous dispersion of oxygen atoms.[4] We have succeeded, after extensive trials of derivative syntheses, in the X-ray crystallographic analysis of a heavy atom-containing analogue obtained from aigialomycin C (**4**). Thus, acylation of **4** with excess 4-bromobenzoyl chloride in pyridine (rt, 3 days) gave a triacyl derivative **14** as a colorless amorphous solid. Selective hydrolysis of **14** under mild basic conditions (aq K_2CO_3, MeOH, rt) gave **15** as a colorless powder, which was subsequently acylated with 3,5-dinitrobenzoyl chloride (in pyridine, rt, 3 days) to obtain the mixed triester **16**. Crystals of **16** were obtained by recrystallization from EtOAc, one of which was subjected to X-ray diffraction analysis. Absolute configuration of this molecule, containing two bromine atoms, was determined to be 1′*R*,2′*R*,5′*S*,6′*S*,10′*S* by the standard anomalous scattering method. Since the only structural difference between compounds **4** and **3** is the oxidation state at C-4′ and the relative configuration at all five carbon centers is identical, the absolute configuration of **3** was assigned to be 1′*R*,2′*R*,4′*S*,5′*S*,6′*S*,10′*S*, and not its mirror image. Taken together with the chemical conversions described above, absolute configuration of both hypothemycin (**1**) and aigialomycin A (**2**) was assigned as 1′*R*,2′*R*,4′*S*,5′*S*,10′*S*, identical to the original proposal for **1** by Nozoe et al.[4]



14: R = 4-bromobenzoyl
15: R = H
16: R = 3,5-dinitrobenzoyl

HRMS and NMR analyses of aigialomycin D (**5**), $C_{19}H_{22}O_8$, revealed that this compound bears an ad-

Table 1. ^{1}H NMR Data of Aigialomycins A–E (2–6)

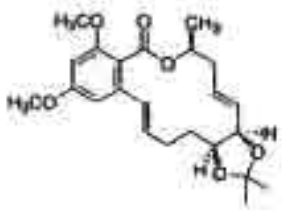
position	2 (CDCl$_3$)	3 (CDCl$_3$)	4 (CDCl$_3$)	5 (acetone-d_6)	6 (acetone-d_6)
3	6.41 (d, 2.8)	6.37 (d, 2.5)	6.40 (d, 2.6)	6.27 (d, 2.4)	6.29 (d, 2.4)
5	6.43 (d, 2.8)	6.39 (d, 2.4)	6.43 (d, 2.6)	6.52 (d, 2.4)	6.19 (d, 2.2)
1′	4.36 (d, 1.7)	4.42 (d, 1.3)	4.39 (s)	7.14 (d, 15.9)	6.70 (d, 11.6)
2′	2.94 (ddd, 9.1, 2.3, 2.1)	2.90 (brd, 8.7)	2.77 (brd, 7.6)	6.09 (ddd, 15.9, 6.6, 5.4)	5.53 (m)
3′	2.04 (ddd, 14.7, 9.1, 2.7)	2.09 (m)	2.26 (m)	2.31–2.34 (m)	2.19 (m)
	1.08 (ddd, 14.7, 9.1, 2.2))	1.51 (m	1.47 (m)	2.31–2.34 (m)	1.40 (m)
4′	4.15 (m)	3.76 (m)	1.90 (m)	2.14 (m)	2.28 (m)
	–	–	1.55 (m)	1.58 (m)	1.78 (m)
5′	4.64 (dd, 3.8, 3.5)	3.71 (d, 8.5)	3.48 (m)	3.82 (m)	3.66 (m)
6′	–	3.99 (dd, 8.9, 8.7)	3.92 (dd, 8.8, 8.7)	4.35 (brd, 4.3)	4.03 (m)
7′	6.40 (d, 15.8)	6.52 (dd, 15.3, 9.2)	5.43 (dd, 15.5, 8.6)	5.69 (dd, 15.7, 5.0)	5.45 (m)
8′	7.02 (ddd, 15.4, 9.8, 6.0)	5.97 (ddd, 15.3, 9.2, 3.4)	5.89 (ddd, 15.5, 4.9, 4.5)	6.87 (dddd, 15.7, 7.3, 7.3, 1.2)	5.43 (m)
9′	2.95 (m)	2.83 (ddd, 16.1, 9.1, 3.8)	2.68 (ddd, 15.2, 9.5, 3.7)	2.55 (ddd, 14.8, 7.6, 3.2)	2.48 (brd, ca. 14)
	2.61 (m)	2.43 (brd, ca. 16)	2.41 (ddd, 15.2, 3.7, 3.7)	2.42 (m)	2.37 (m)
10′	5.57 (m)	5.60 (m)	5.55 (m)	5.42 (m)	5.22 (m)
10′-CH_3	1.48 (d, 6.6)	1.43 (d, 6.4)	1.44 (d, 6.6)	1.39 (d, 6.4)	1.39 (d, 6.2)
4-OCH_3	3.90 (s)	3.77 (s)	3.80 (s)	–	–
2-O*H*	11.61 (s)	11.67 (s)	11.93 (s)	11.65 (s)	11.7 (br)
4-O*H*	–	–	–	9.5 (br)	9.5 (br)
4′-O*H*	2.48 (brd, 9.7)	3.43 (br)	–	–	–
5′-O*H*	3.81 (br)	4.20 (br)	2.0 (br)	not detected	not detected
6′-O*H*	–	3.58 (br)	2.8 (br)	not detected	not detected

Table 2. ^{13}C NMR Data of Aigialomycins A–E (2–6)

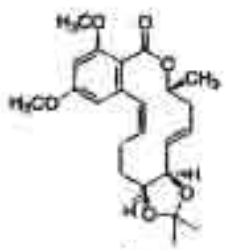
position	2 (CDCl$_3$)	3 (CDCl$_3$)	4 (CDCl$_3$)	5 (acetone-d_6)	6 (acetone-d_6)
1	104.4 (s)	104.6 (s)	104.2 (s)	104.3 (s)	102.4 (s)
2	165.5 (s)	165.3 (s)	165.7 (s)	165.5 (s)	162.9 (s)
3	101.0 (d)	100.7 (d)	100.8 (d)	102.4 (d)	102.3 (d)
4	165.0 (s)	164.8 (s)	164.8 (s)	163.1 (s)	162.6 (s)
5	104.1 (d)	104.1 (d)	104.5 (d)	107.7 (d)	111.6 (d)
6	141.9 (s)	142.0 (s)	142.3 (s)	144.2 (s)	143.2 (s)
1′	56.2 (d)	56.8 (d)	55.5 (d)	130.6 (d)	131.4 (d)
2′	62.5 (d)	62.3 (d)	63.4 (d)	133.6 (d)	131.7 (d)
3′	34.9 (t)	33.2 (t)	25.8 (t)	27.9 (t)	26.7 (t)
4′	71.1 (d)	69.8 (d)	28.6 (t)	26.5 (t)	31.8 (t)
5′	78.4 (d)	77.2 (d)	72.9 (d)	73.1 (d)*	72.3 (d)
6′	197.2 (s)	74.8 (d)	77.1 (d)	76.4 (d)	77.9 (d)
7′	129.3 (d)	131.4 (d)	133.7 (d)	135.6 (d)	135.5 (d)
8′	143.6 (d)	129.6 (d)	128.3 (d)	125.4 (d)	127.7 (d)
9′	37.8 (t)	36.7 (t)	36.4 (t)	37.9 (t)	39.9 (t)
10′	71.0 (d)	71.0 (d)	71.6 (d)	72.9 (d)*	72.3 (d)
-COO-	170.9 (s)	170.8 (s)	170.9 (s)	172.1 (s)	175.8 (s)
10′-CH_3	19.0 (q)	19.0 (q)	18.8 (q)	19.1 (q)	20.7 (q)
4-OCH_3	55.5 (q)	55.4 (q)	55.4 (q)	–	–

* Assignments can be interchanged.

ditional *trans*-olefin at C-1′–C-2′ ($J_{1',2'}$ = 15.9 Hz) instead of the epoxide functionality, and also free, instead of the methylated, 4-OH group on the aromatic nucleus. The *cis*-fused acetonide structure was assigned by NOESY analyses for the derivative **17** ($J_{3',8'}$ = 5.4 Hz), synthesized from **5**. Hence, structure of aigialomycin D (**5**) was straightforwardly identified.



17



18

Aigialomycin E (**6**) has the same molecular formula to that of **5**. NMR analyses of **6** revealed a similar structural feature to that of **5**, but it differed at the olefinic geometry at C-1′–C-2′, having a *cis* ($J_{1',2'}$ = 11.6 Hz) relationship. The *trans* geometry of the C-7′–C-8′ double bond was not clear in the ^{1}H NMR spectrum of **6** due to signals overlapping. However, the ^{1}H NMR spectrum of acetonide derivative **18**, synthesized from **6**, showed vicinal ^{1}H–^{1}H coupling constants of $J_{7',8'}$ = 11.5 Hz and $J_{7',8'}$ = 15.4 Hz. These data confirmed the *cis* C-1′–C-2′ and *trans* C-7′–C-8′ double bonds' geometries. NOESY spectral analyses indicated the *cis*-acetonide structure of **18** while correlations and vicinal ^{1}H–^{1}H coupling constants revealed the relative configuration between the *cis*-diol moiety and the C-10′ asymmetric center, as well as the probable conformation of **18** (Figure 2). Thus, the large $J_{6',7'}$ value of 9.1 Hz and the intense NOESY correlations between H-6′ and H-8′ as well as the weak intensity of the cross signal between H-6′ and H-7′ were observed. These indicated the antiperiplanar relationship of H-6′ and H-7′ where the *trans*-olefin is vertical to the acetonide ring. H-7′ correlated to one of the H-9′ methylene protons at δ_H 2.33 and correlations to another H-9′ (at δ_H 2.42) from both H-8′ and H-10′ were also evident. Intense NOESY cross signal was also found between H-8′ and H-10′. The δ_H 2.33 proton was coupled to H-8′ with the J value of 9.8 Hz, while the NOESY cross signal between these protons was very weak indicating the

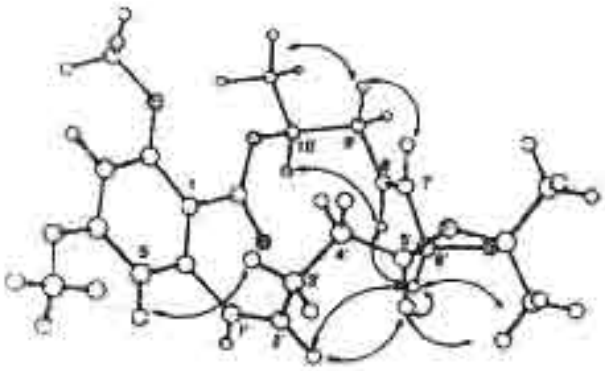


Figure 2. Probable conformation of compound **18**. Selected NOESY correlations are illustrated with solid arrows.

antiperiplanar relationship of these two protons. These NMR data placed H-6′, H-8′, and H-10′ on the same side of the macrolide ring, suggesting an approximate conformation from C-5′ to C-10′ in the molecule. Other important NOESY correlations were that of H-2′ to both H-5′ and H-6′. The structure shown in Figure 2 indicates approximate conformation for the 5′*S*,6′*R*,10′*S* isomer of **18**. Conformational studies using molecular models confirmed the above arguments. Based upon the absolute configuration of aigialomycin C (**4**), the absolute stereochemistry of aigialomycin E (**6**) should be assigned as 5′*S*,6′*R*,10′*S* rather than its mirror image (5′*R*,6′*S*,10′*R*). Aigialomycin D (**5**) should also possess the same sense of absolute configuration as **6**.

Several 14-membered resorcylic macrolides have previously been isolated;[5] e.g., zearalenone,[5a] radicicol,[5b] zeaenol,[5c] LL-Z-1640s,[5d] monocillines,[5e] nordinone,[5f] and nordinonediol.[5g] Hypothemycin and aigialomycins A and B are highly oxygenated analogues in this class. The structure of aigialomycin E, having a *cis*-olefin at C-1′-C-2′, may be of remarkable interest, since all of the previously known compounds possess either a *trans*-olefinic or saturated bond at these two carbons.

Hypothemycin (**1**) and aigialomycins A–E (**2**–**6**) were subjected to antimalarial activity screening against *Plasmodium falciparum* K1. Cytotoxicities of these compounds against two cancer cells (KB, BC-1) and Vero cells (African green monkey kidney fibroblast) were also determined (Table 3). Hypothemycin (**1**) and aigialomycin D (**5**) exhibited moderate antimalarial activity, while other congeners were inactive at a concentration of 20 μg/mL. It should be noted that compounds **1** and **5** also showed cytotoxicity; therefore, the activity of these compounds against *P. falciparum* may well be related to their cytotoxic activity. Hypothemycin is reported to exhibit moderate antibiotic activity against the protozoan, *Tetrahymena furgasoni*, and the plant pathogenic fungi *Ustilago maydis* and *Botrytis allii*.[5] It is also known to be cytotoxic against P388, L1210, Colon 26, A549, and DLD-1 with IC_{50} values of 0.252–1.50 μg/mL.[4]

(5) (a) Urry, W. H.; Wehrmeister, H. L.; Hodge, E. B.; Hidy, P. H. *Tetrahedron Lett.* **1966**, 3109–3114. (b) Mirrington, R. N.; Ritchie, E.; Shoppee, C. W.; Taylor, W. C.; Sternhall, S. *Tetrahedron Lett.* **1964**, 365–370. (c) Sugawara, F.; Kim, K.-W.; Kobayashi, K.; Uzawa, J.; Yoshida, S.; Murofushi, N.; Takahashi, N.; Strobel, G. A. *Phytochemistry* **1992**, *31*, 1987–1990. (d) Ellestad, G. A.; Lovell, F. M.; Perkinson, N. A.; Hargreaves, R. T.; McGahren, W. J. *J. Org. Chem.* **1978**, *43*, 2339–2343. (e) Ayer, W. A.; Lee, S. P.; Tsuneda, A.; Hiratsuka, Y. *Can. J. Microbiol.* **1980**, *26*, 766–773. (f) Ayer, W. A.; Pena-Rodrigues, L. *Phytochemistry* **1987**, *26*, 1353–1355.

Table 3. Antimalarial Activity and Cytotoxicity of Macrolides 1–6

compound	*P. falciparum* K1 (IC_{50}, μg/mL)	cytotoxicity (IC_{50} μg/mL)[a] KB	BC-1	Vero
hypothemycin (**1**)	2.2	17	6.2	6.3
aigialomycin A (**2**)	>20	>20	11	4.3
aigialomycin B (**3**)	>20	>20	>20	>20
aigialomycin C (**4**)	>20	>20	>20	>20
aigialomycin D (**5**)	6.6	3.0	18	1.8
aigialomycin E (**6**)	>20	>20	15	>20
chloroquine diphosphate[a]	0.16	16	>20	>20

[a] Standard antimalarial compound. [b] IC_{50} values of a standard compound ellipticine are 0.46 μg/mL for KB cells, 0.60 μg/mL for BC-1 cells, and 1.0 μg/mL for Vero cells.

Experimental Section

Isolation of Macrolides. *A. pannus* was collected, identified, and isolated from mangrove wood by Prof. E. B. G. Jones. This fungus is deposited at the BIOTEC Culture Collection as BCC 5311. An isolated culture of the strain BCC 5311 was grown on potato dextrose agar (PDA) at 22 °C for 35 days, before inoculation into 56 × 1 L Erlenmeyer flasks each containing 250 mL of potato dextrose broth (PDB). After static incubation at 22 °C for 35 days, the flask cultures were filtered, and the filtrate (14 L) was extracted with equal volume of EtOAc to obtain a light brown solid (1.4 g) after solvent evaporation. The crude extract was passed through a Sephadex LH-20 column chromatography (3.5 × 25 cm) using MeOH/CH_2Cl_2 = 75:25 as an eluent to obtain a mixture of macrolides. This mixture was subjected to column chromatography on silica gel (3.5 × 15 cm; step gradient elution with MeOH/CH_2Cl_2, 2:98 to 30:70). The following fractions were obtained in the order of elution: Fr-A, compounds **2** (major) and **1**, 136 mg; Fr-B, compounds **2**, **1** (major), and **4**, 661 mg; Fr-C, compounds **3**, **5** and **6**, 133 mg. Fr-A was subjected to preparative HPLC using a reversed-phase column (Prep Nova-Pak HR C_{18}, 6 μM, 40 × 100 mm) with MeCN/H_2O = 50:50 as an eluent at a flow rate of 20 mL/min to obtain **2** (61 mg) and **1** (22 mg). Fr-B was triturated twice in MeOH (5 mL, room temp, 24 h) to obtain colorless crystals of **1** (283 mg), and the dried filtrate (354 mg) was subjected to prep HPLC (MeOH/H_2O = 50:50) to yield **2** (40 mg), **1** (73 mg) and **4** (33 mg). Fr-C was chromatographed on silica gel (1.2 × 15 cm, MeOH/EtOAc, step gradient elution, 0:100 to 5:95) to obtain a mixture of **5** and **6** (50 mg), and **3** (40 mg). Compounds **5** and **6** were separated by prep HPLC (MeOH/H_2O = 40:60): **5** (25 mg); **6** (7.5 mg). Compound **3** was further purified by prep HPLC (yield, 28 mg).

Hypothemycin (1). Colorless prisms (MeOH): mp 170–172 °C; $[\alpha]^{28}_D$ −18 (*c* 0.50, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 220 (4.53), 267 (4.12), 308 (3.80) nm; IR (KBr) ν_{max} 3390, 1685, 1645, 1619, 1576, 1254, 1048 cm^{-1}; MS (ESI-TOF) *m/z* 401 [M + Na]$^+$, 379 [M + H]$^+$, 361, 343, 253, 235; ^1H and ^{13}C NMR data were identical to those reported in the literature.[4] Anal. C 60.43%, H 5.79%, calcd for $C_{18}H_{22}O_8$, C 60.31%, H 5.86%.

Aigialomycin A (2). Colorless crystals: mp 166–168 °C (dec); $[\alpha]^{25}_D$ +17 (*c* 0.50, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 220 (4.50), 266 (4.04), 306 (3.74) nm; IR (KBr) ν_{max} 3509, 1668, 1645, 1623, 1580, 1261, 1044 cm^{-1}; MS (ESI-TOF) *m/z* 401 [M + Na]$^+$, 379 [M + H]$^+$, 361, 255, 218; HRMS (ESI-TOF) *m/z* [M + H]$^+$ 379.1397 (calcd for $C_{18}H_{23}O_8$, 379.1393); ^1H and ^{13}C NMR data, see Tables 1 and 2. Anal. C 60.46%, H 5.87%, calcd for $C_{18}H_{22}O_8$, C 60.31%, H 5.86%.

Aigialomycin B (3). Colorless crystals: mp 82–84 °C; $[\alpha]^{24}_D$ −10 (*c* 0.27, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 220 (4.37), 266 (4.28), 306 (3.74) nm; IR (KBr) ν_{max} 3403, 1651, 1616, 1576, 1256, 1046 cm^{-1}; MS (ESI-TOF) *m/z* 381 [M + H]$^+$, 269, 256; HRMS (ESI-TOF) *m/z* [M + H]$^+$ 381.1548 (calcd for $C_{18}H_{25}O_8$, 381.1549); ^1H and ^{13}C NMR data, see Tables 1 and 2.

Aigialomycin C (4). Colorless amorphous solid: $[\alpha]^{23}_D$ −20 (*c* 0.25, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 219 (4.45), 265 (4.14),

306 (3.82) nm; IR (KBr) ν_{max} 3419, 1646, 1615, 1575, 1318, 1257, 1204, 1160, 1041, 966 cm^{-1}; MS (ESI-TOF) *m/z* 365 [M + H]$^+$, 347, 288; HRMS (ESI-TOF) *m/z* [M + H]$^+$ 365.1617 (calcd for $C_{19}H_{25}O_7$, 365.1600); ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

Aigialomycin D (5). Colorless crystals: mp 83–85 °C; $[\alpha]^{24}_D$ −19 (*c* 0.24, MeOH); UV (MeOH) λ_{max} (log ϵ) 238 (4.42), 275 (4.05), 314 (3.71) nm; IR (KBr) ν_{max} 3336, 1640, 1602, 1315, 1262, 1165, 1013, 968 cm^{-1}; MS (EI) *m/z* 334 [M]$^+$, 316, 237, 219, 208, 189, 175, 163; HRMS (ESI-TOF) *m/z* [M + H]$^+$ 335.1499 (calcd for $C_{18}H_{23}O_6$, 335.1494); ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

Aigialomycin E (6). Colorless amorphous solid: mp 91–94 °C; $[\alpha]^{24}_D$ +14 (*c* 0.28, MeOH); UV (MeOH) λ_{max} (log ϵ) 226 (4.31), 268 (4.00), 307 (3.69) nm; IR (KBr) ν_{max} 3401, 1646, 1615, 1318, 1261, 1163, 1021, 969 cm^{-1}; MS (EI) *m/z* 334 [M]$^+$, 316, 237, 219, 189, 175; HRMS (ESI-TOF) *m/z* [M + H]$^+$ 335.1498 (calcd for $C_{18}H_{23}O_6$, 335.1494); ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

Hydrogenation of 1. To a solution of hypothemycin (1, 30 mg) in EtOAc (4 mL) was added 10% Pd/C (20 mg), and the mixture was stirred under hydrogen for 26 h. The suspension was filtered by suction, and the filtrate was concentrated and subjected to column chromatography on silica gel (MeOH/CH_2Cl_2) to obtain dihydrohypothemycin **7** (25.4 mg, 84%). Recrystallization from MeOH gave colorless crystals. Hydrogenation of compound **2** (20 mg) under the same reaction conditions gave a compound (13.5 mg, 67%) whose spectral data were identical to **7**.

Dihydrohypothemycin (7). Colorless crystals: mp 175–177 °C; $[\alpha]^{26}_D$ −16 (*c* 0.17, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 218 (4.44), 265 (4.14), 305 (3.81) nm; IR (KBr) ν_{max} 3404, 1698, 1650, 1618, 1256, 1159, 1051, 1023 cm^{-1}; HRMS (ESI-TOF) *m/z* [M + H]$^+$ 381.1541 (calcd for $C_{18}H_{21}O_8$, 381.1549); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.98 (1H, s, 2-OH), 6.50 (1H, d, J = 2.6 Hz, H-5), 6.41 (1H, d, J = 2.5 Hz, H-3), 5.26 (1H, m, H-10′), 4.52 (1H, brs, H-5′), 4.42 (1H, d, J = 1.4 Hz, H-1′), 4.22 (1H, brd, J = 10.5 Hz, H-4′), 3.81 (3H, s, 4-OCH_3), 3.58 (1H, brs, 5′-OH), 2.98 (1H, d, J = 8.5 Hz, H-2′), 2.82 (1H, m, H-7′a), 2.42 (1H, m, H-7′b), 2.38 (1H, brs, 4′-OH), 2.16 (1H, dd, J = 15.4, 10.9 Hz, H-3′a), 1.80–1.64 (4H, m, H-8′ and H-9′), 1.39 (3H, d, J = 6.3 Hz, 10′-CH_3), 1.20 (1H, dd, J = 15.5, 9.5 Hz, H-3′b); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 208.3 (s, C-6′), 170.9 (s, -COO-), 165.9 (s, C-4), 165.1 (s, C-2), 142.0 (s, C-6), 104.0 (s, C-1), 103.9 (d, C-5), 101.0 (d, C-3), 82.2 (d, C-5′), 73.0 (d, C-10′), 69.7 (d, C-4′), 61.1 (d, C-2′), 58.3 (d, C-1′), 55.5 (q, 4-OCH_3), 38.5 (t, C-7′), 34.6 (t, C-3′), 34.5 (t, C-9′), 21.2 (t, C-8′), 20.5 (q, 10′-CH_3).

Preparation of Acetonides 10 and 11. To a solution of aigialomycin B (3; 6.0 mg) in 2,2-dimethoxypropane (1 mL) was added a very small amount of *p*-TsOH·H_2O. After stirring at room temperature for 3 h, saturated aq $NaHCO_3$ was added and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and concentrated in vacuo to obtain an acetonide mixture (**8** and **9**; 7.3 mg). The crude acetonide mixture was dissolved in 2-butanone (0.3 mL), MeI (50 μL) and K_2CO_3 (s) (50 mg) were added, and the mixture was stirred for 34 h. After standard aqueous workup, the crude product (7.9 mg) was subjected to preparative reversed-phase HPLC (40 × 100 mm; MeCN/H_2O = 35:65; 20 mL/min) to obtain compounds **10** (4.2 mg; t_R 22 min) and **11** (2.3 mg; t_R 16 min).

Acetonide 10. Colorless amorphous solid: IR (KBr) ν_{max} 3486, 1722, 1605, 1460, 1262, 1161, 1045 cm^{-1}; HRMS (ESI-TOF) *m/z* [M + H]$^+$ 435.2032 (calcd for $C_{23}H_{31}O_8$, 435.2019); ^{1}H NMR ($CDCl_3$, 400 MHz) selected signals, δ 4.52 (1H, dd, J = 7.7, 7.7 Hz, H-6′), 4.18 (1H, brd, J = ca 12 Hz, H-4′), 3.84 (1H, m, H-5′), 1.45 (6H, s, acetonide CH_3 × 2); ^{1}H NMR (methanol-d_4, 400 MHz) δ 6.58 (1H, d, J = 2.2 Hz, H-3), 6.37 (1H, d, J = 2.2 Hz, H-5), 6.02 (1H, ddd, J = 15.7, 6.4, 5.5 Hz, H-8′), 5.74 (1H, dd, J = 15.7, 7.4 Hz, H-7′), 5.61 (1H, m, H-10′), 4.53 (1H, dd, J = 7.7, 7.7 Hz, H-6′), 4.19 (1H, ddd, J = 11.5, 3.8, 1.5 Hz, H-4′), 3.94 (1H, d, J = 1.6 Hz, H-1′), 3.85 (3H, s, 2-OCH_3 or 4-OCH_3), 3.84 (3H, s, 4-OCH_3 or 2-OCH_3), 3.78 (1H, dd, J = 8.0, 1.6 Hz, H-5′), 2.88 (1H, m, H-2′), 2.57 (1H, m, H-9′a), 2.47 (1H, m, H-9′b), 2.28 (1H, ddd, J = 14.2, 3.5, 3.3 Hz, H-3′a), 1.57 (1H, ddd, J = 14.0, 11.6, 8.8 Hz, H-3′b), 1.45 (3H, s, acetonide), 1.42 (3H, s, acetonide), 1.40 (3H, d, J = 6.5 Hz, 10′-CH_3).

Acetonide 11. Colorless amorphous solid: IR (KBr) ν_{max} 3484, 1723, 1606, 1460, 1261, 1160, 1043 cm^{-1}; HRMS (ESI-TOF) *m/z* [M + H]$^+$ 435.2034 (calcd for $C_{23}H_{31}O_8$, 435.2019); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.39 (2H, brs, H-3 and H-5), 5.97 (1H, ddd, J = 16.0, 6.5, 6.1 Hz, H-8′), 5.68 (1H, dd, J = 16.0, 7.2 Hz, H-7′), 5.41 (1H, m, H-10′), 4.27 (1H, m, H-4′), 4.13 (1H, dd, J = 9.5, 7.2 Hz, H-6′), 4.07 (1H, dd, J = 9.6, 5.0 Hz, H-5′), 3.88 (1H, d, J = 1.7 Hz, H-1′), 3.81 (3H, s, 2-OCH_3 or 4-OCH_3), 3.79 (3H, s, 4-OCH_3 or 2-OCH_3), 2.87 (1H, m, H-2′), 2.68 (1H, brs, 6′-OH), 2.48 (1H, m, H-9′a), 2.42 (1H, m, H-9′b), 2.02 (1H, m, H-3′a), 1.98 (1H, m, H-3′b), 1.49 (3H, s, acetonide), 1.39 (3H, d, J = 6.4 Hz, 10′-CH_3), 1.37 (3H, s, acetonide).

Acetonide 12. Colorless powder: HRMS (ESI-TOF) *m/z* [M + H]$^+$ 419.1724 (calcd for $C_{22}H_{27}O_8$, 419.1706); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.76 (1H, s, 2-OH), 6.96 (1H, ddd, J = 16.4, 7.2, 7.1 Hz, H-8′), 6.50 (1H, d, J = 2.6 Hz, H-5), 6.42 (1H, d, J = 2.6 Hz, H-3), 6.27 (1H, d, J = 16.4 Hz, H-7′), 5.55 (1H, m, H-10′), 4.97 (1H, d, J = 6.3 Hz, H-5′), 4.54 (1H, ddd, J = 6.4, 6.4, 5.4 Hz, H-4′), 4.34 (1H, d, J = 1.6 Hz, H-1′), 3.80 (3H, s, 4-OCH_3), 2.83 (1H, m, H-9′a), 2.66 (1H, m, H-2′), 2.61 (1H, m, H-9′b), 2.14 (1H, m, H-3′a), 1.80 (1H, ddd, J = 15.1, 7.0, 6.8 Hz, H-3′b), 1.63 (3H, s, acetonide), 1.46 (3H, d, J = 6.5 Hz, 10′-CH_3), 1.39 (3H, s, acetonide).

Acetonide 13. Colorless powder: HRMS (ESI-TOF) *m/z* [M + H]$^+$ 419.2007 (calcd for $C_{23}H_{31}O_7$, 419.1992); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.41 (1H, d, J = 2.3 Hz, H-3), 6.30 (1H, d, J = 2.3 Hz, H-5), 6.01 (1H, ddd, J = 15.7, 7.3, 4.7 Hz, H-8′), 5.71 (1H, dd, J = 15.8, 6.7 Hz, H-7′), 5.61 (1H, m, H-10′), 4.01 (1H, dd, J = 7.5, 7.3 Hz, H-6′), 3.85 (1H, d, J = 1.7 Hz, H-1′), 3.81 (3H, s, 2-OCH_3), 3.79 (3H, s, 4-OCH_3), 3.72 (1H, ddd, J = 8.1, 8.1, 4.8 Hz, H-5′), 2.84 (1H, ddd, J = 6.9, 2.2, 2.1 Hz, H-2′), 2.48 (1H, m, H-9′a), 2.40 (1H, m, H-9′b), 2.17 (1H, m, H-3′a), 1.94 (1H, m, H-4′a), 1.73 (1H, m, H-4′b), 1.55 (1H, m, H-3′b), 1.43 (3H, s, acetonide), 1.41 (3H, s, acetonide), 1.37 (3H, d, J = 6.3 Hz, 10′-CH_3).

Acetonide 17. Colorless powder: HRMS (ESI-TOF) *m/z* [M + H]$^+$ 403.2126 (calcd for $C_{22}H_{31}O_8$, 403.2120); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.56 (1H, d, J = 1.9 Hz, H-5), 6.35 (1H, d, J = 1.9 Hz, H-3), 6.26 (1H, d, J = 15.4 Hz, H-1′), 6.14 (1H, ddd, J = 15.3, 9.9, 4.2 Hz, H-2′), 5.73 (1H, ddd, J = 15.2, 9.3, 3.5 Hz, H-8′), 5.59 (1H, ddd, J = 15.2, 9.6, 1.6 Hz, H-7′), 5.32 (1H, m, H-10′), 4.57 (1H, dd, J = 9.6, 5.4 Hz, H-6′), 4.19 (1H, ddd, J = 11.6, 5.3, 3.1 Hz, H-5′), 3.82 (3H, s, 2-OCH_3), 3.79 (3H, s, 4-OCH_3), 2.51 (1H, m, H-9′a), 2.47 (1H, m, H-9′b), 2.30 (1H, m, H-3′a), 2.10 (1H, m, H-4′a), 1.82 (1H, m, H-3′a), 1.53 (1H, m, H-4′b), 1.47 (3H, s, acetonide), 1.36 (3H, d, J = 6.1 Hz, 10′-CH_3), 1.35 (3H, s, acetonide).

Acetonide 18. Colorless powder: HRMS (ESI-TOF) *m/z* [M + H]$^+$ 403.2134 (calcd for $C_{22}H_{31}O_8$, 403.2120); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.47 (1H, d, J = 11.5 Hz, H-1′), 6.36 (1H, d, J = 2.0 Hz, H-3), 6.29 (1H, d, J = 1.8 Hz, H-5), 5.74 (1H, ddd, J = 11.1, 11.0, 5.5 Hz, H-2′), 5.57 (1H, ddd, J = 15.4, 9.8, 3.9 Hz, H-8′), 5.43 (1H, ddd, J = 15.3, 9.1, 1.0 Hz, H-7′), 5.20 (1H, m, H-10′), 4.49 (1H, dd, J = 9.0, 5.8 Hz, H-6′), 4.15 (1H, ddd, J = 8.0, 5.5, 4.3 Hz, H-5′), 3.81 (6H, s, 2-OCH_3 and 4-OCH_3), 2.63 (1H, m, H-3′a), 2.42 (1H, m, H-9′a), 2.33 (1H, m, H-9′b), 1.96 (1H, m, H-3′b), 1.64 (1H, m, H-4′a), 1.55 (1H, m, H-4′b), 1.43 (3H, s, acetonide), 1.37 (3H, d, J = 6.3 Hz, 10′-CH_3), 1.35 (3H, s, acetonide).

Synthesis of Compound 16. To a solution of aigialomycin C (4; 3.0 mg) in pyridine (0.3 mL) was added excess *p*-bromobenzoyl chloride (10 mg), and the mixture was stirred at room temperature for 2 days. After the usual aqueous workup, the crude mixture was subjected to preparative HPLC (reversed-phase column; MeCN/H_2O = 80:20, then 100:0) to obtain the triester **14** (4.6 mg). This product was treated in aq K_2CO_3 (0.2 mL)/dioxane (0.4 mL)/MeOH (0.2 mL) for 6 h and then concentrated in vacuo. Standard aqueous workup and purification by column chromatography on silica gel (CH_2Cl_2) afforded compound **15** (3.0 mg). A portion of **15** (1.0 mg) was treated with 3,5-dinitrobenzoyl chloride (10 mg) in pyridine (0.15 mL) for 2 days. The usual aqueous workup and subse-

quest CC on silica gel gave the mixed ester **16** (0.5 mg). Recrystallization in EtOAc gave colorless crystals: MS (ESI-TOF) *m/z* 945, 947 and 949 $[M + Na]^+$; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.31 (1H, t, J = 1.9 Hz), 8.29 (2H, d, J = 1.8 Hz), 7.79 (2H, d, J = 8.4 Hz), 7.75 (2H, d, J = 8.5 Hz), 7.52 (2H, d, J = 8.4 Hz), 7.48 (2H, d, J = 8.5 Hz), 6.84 (1H, d, J = 2.4 Hz), 6.76 (1H, d, J = 2.2 Hz), 6.25 (1H, m), 5.78 (1H, dd, J = 9.3, 9.1 Hz), 5.65 (1H, dd, J = 15.8, 9.3 Hz), 5.48 (1H, m), 5.23 (1H, m), 4.23 (1H, brs), 3.86 (3H, s), 2.81 (1H, brd, J = 9.2 Hz), 2.61 (1H, m), 2.46 (1H, m), 2.29 (1H, m), 2.14 (1H, m), 1.72 (1H, m), 1.47 (1H, m), 1.40 (3H, d, J = 6.3 Hz).

Single-Crystal X-ray Diffraction Analysis of 16. Crystallographic data: $C_{40}H_{32}N_2O_{14}Br_2$, MW 924.50, monoclinic, $P2_1$, a = 13.0176(4) Å, b = 9.1772(3) Å, c = 13.7093(5) Å, and β = 104.034(2)°, V = 2168.4(1) Å^3, Z = 2, D_x = 1.465 g/cm^{-1}. The asymmetric unit contained one molecule of **16** and one disordered solvated methanol. Absolute configuration of **16** was established by using anomalous scattering methods (Flack parameter = +0.069(1) and 0.885(2) for the inverted structure). Of the 7183 unique reflections, the 6768 reflections with $F > 4\sigma(F)$ were used in the least-squares refinement to yield R = 0.0623 and R_w = 0.1661. The atomic coordinates have been deposited at the Cambridge Crystallographic Data Center (CCDC 168877). Copies of the data can be obtained, on request, from the Director at Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK.

Biological Assays. The assay for activity against *P. falciparum* K1 was performed using the protocol previously reported[6] which follows the microculture radioisotope technique described by Desjardins.[7] IC_{50} represents the concentration that causes 50% reduction of parasite growth as indicated by the in vitro uptake of [^{3}H]-hypoxanthine by *P. falciparum*. Cytotoxicity of the purified compounds against human epidermoid carcinoma (KB cells), human breast cancer cells (BC-1 cells), and African green monkey kidney fibroblast (Vero cells) were tested using the colorimetric method.[8]

Acknowledgment. Financial support from the Biodiversity Research and Training Program (BRT) is gratefully acknowledged. BIOTEC antimalarial screening laboratory was partly supported by Thailand-Tropical Diseases Research Program (T-2). We are also grateful to Dr. Emil Lobkovsky for his kind assistance in X-ray data collection. A Senior Research Fellowship Award from BIOTEC/NSTDA to Y. T. and a Research Scholar Award from the Thailand Research Fund (TRF) to P. K. are acknowledged.

Supporting Information Available: Detailed information of the X-ray crystallographic analysis of compound **16** including structure diagram, crystal data, refinement, atomic coordinates, isotropic displacement parameters, bond lengths, and angles. This material is available free of charge via the Internet at http://pubs.acs.org.

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Appendix III

Reprint

P. Kongsaeree, C. Samanchart, P. Laowanapiban, S. Wiyakrutta, and V. Meevootisom.
Crystallization and preliminary X-ray crystallographic analysis of
D-phenylglycine aminotransferase from *Pseudomonas stutzeri* ST201.
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Crystallization and preliminary X-ray crystallographic analysis of D-phenylglycine aminotransferase from *Pseudomonas stutzeri* ST201

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D-Phenylglycine aminotransferase (D-PhgAT) catalyzes the reversible transamination of D-phenylglycine to L-glutamate with 2-oxoglutarate as the amino-group acceptor. Crystals of substrate-free *Pseudomonas stutzeri* D-PhgAT bound to the cofactor pyridoxal-5′-phosphate (PLP) were obtained by the hanging-drop vapour-diffusion method using ammonium sulfate as a precipitant. The crystals belong to space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 75.155$, $c = 147.554$ Å. The asymmetric unit contains one molecule of D-PhgAT and has a solvent content of 50.0%. A complete native X-ray diffraction data set was collected from a single crystal at 100 K to a resolution of 2.3 Å.

1. Introduction

D-Phenylglycine aminotransferase (D-PhgAT), a vitamin B_6-dependent enzyme, catalyzes the stereospecific amino-group transfer of D-phenylglycine to 2-oxoglutarate to yield L-glutamate by a reversible ping-pong kinetic mechanism (Wiyakrutta & Meevootisom, 1997; Kirsch *et al.*, 1984). The enzyme requires pyridoxal phosphate (PLP) as a coenzyme and has substrate specificity for D-phenylglycine and D-4-hydroxyphenylglycine. However, neither D- nor L-aromatic nor branch-chained amino acids can be utilized as substrates. Using the 'stereo-inverting' transamination property of D-PhgAT, the amino nitrogen can be channelled directly between D-phenylglycine and L-glutamate, a central molecule in cellular nitrogen metabolism, without the need for additional amino-acid racemases for phenylglycine or glutamate. D-PhgAT, found as a homodimer of molecular mass 92 kDa, has been purified from *Pseudomonas stutzeri* ST201 (Wiyakrutta & Meevootisom, 1997). The enzyme is most active at alkaline pH, with maximum activity at pH 9–10, and can be inhibited by typical inhibitors of pyridoxal phosphate-dependent enzymes. The D-PhgAT encoding gene, isolated from the plasmid pBPL-ph, was cloned into the expression vector pET-17b and the expression host BL21(DE3) (Laowanapiban, 2001). From a BLASTp search (Altschul *et al.*, 1997) using the deduced amino-acid sequence, the 453-residue D-PhgAT was found to be homologous to the aminotransferase subgroup II (Mehta *et al.*, 1993) or aminotransferase subclass II (Schneider *et al.*, 2000), including glutamate-1-semialdehyde aminomutase (GSAAT) and ornithine aminotransferase (OAT) with sequence identity of 25, 30 and 21%, respectively. Notably, D-PhgAT lacks sequence homology to D-amino-acid aminotransferase (DAAT), having a sequence identity of only 16%.

In general, the amino-group transfer reaction catalyzed by aminotransferases is highly stereo-conserved (Sugio *et al.*, 1995; Peisach *et al.*, 1998) and there is little information regarding transamination reactions between two amino acids which have opposite configuration (van den Tweel *et al.*, 1986, 1988). Therefore, we have initiated a crystallographic investigation of the stereospecific reaction mechanism, as well as of the substrate specificity of the D-PhgAT enzyme.

2. Experimental

2.1. Overexpression and purification

pEPL-transformed *E. coli* BL21(DE3) cells were grown in LB medium with 50 mg l^{-1} ampicillin at 305 K. When the culture reached an absorbance at 600 nm of 2.0, IPTG was added to 0.4 m*M* and the culture was further incubated for 3 h. The cells were harvested by centrifugation, resuspended in 50 m*M* phosphate buffer pH 7.0 with 1 m*M* PLP and 1 m*M* PMSF and subsequently disrupted by a sonicator. The protein was purified by ammonium sulfate precipitation at 25–45% saturation, followed by chromatography on a phenyl-agarose CL-4B column and a DEAE anion-exchange column. The combined active fractions were desalted and concentrated to about 10 mg ml^{-1} using a Centricon Plus-20 ultrafiltration membrane (Amicon, Beverly, MA, USA).

2.2. Crystallization and X-ray data collection

Initial crystallization conditions were obtained using the vapour-diffusion technique

Figure 1
Photograph of a recombinant D-PhgAT trigonal crystal (approximate dimensions 0.2 × 0.2 × 0.2 mm)

in a 24-well tissue-culture plate at 277 and 298 K. In each drop, 2 µl of 10 mg ml^{-1} D-PhgAT solution in 10 m*M* Tris pH 7.8, 100 m*M* NaCl, 1 m*M* EDTA was mixed with an equal volume of reservoir solution. Promising conditions were discovered using $(NH_4)_2SO_4$ and pH-screening grids at 277 K and were further optimized with respect to pH and precipitant concentration. The optimal condition was found to consist of 200 m*M* phosphate buffer pH 6.2 and 28–30% saturated $(NH_4)_2SO_4$. These rhombohedral crystals typically grew to 0.2 × 0.2 × 0.2 mm within a few weeks (Fig. 1).

2.3. Data collection

A 2.3 Å resolution data set from D-PhgAT in complex with PLP was collected at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory on beamline X8C. A single crystal (0.1 × 0.1 × 0.1 mm), cryoprotected by a 60 s soak in a reservoir solution containing 35% $(NH_4)_2SO_4$, 200 m*M* phosphate buffer pH 6.2 and 30% glycerol, was flash-frozen in the cryogenic nitrogen stream using a nylon loop. The crystals of D-PhgAT complexed with PLP possessed trigonal symmetry, with unit-cell parameters $a = b = 75.155$, $c = 149.554$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. The reflection data were collected on an ADSC Quantum-4 CCD detector using X-ray radiation ($\lambda = 0.9795$ Å) with a crystal-to-detector distance of 200 mm and a 0.5° oscillation angle per image, with an exposure time of 60 s for each image and a total of 360 frames (Fig. 2). The diffraction data were integrated, scaled and reduced with *MOSFLM* and *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994) (Table 1). The Laue symmetry and systematic absences showed a clear threefold screw axis, identifying the space group as either $P3_121$ or $P3_221$. The data set was 99.8% complete, with a scaling R_{sym} of 0.065 for 22 083 unique reflections in the resolution range 20.0–2.3 Å (Table 2). Although D-PhgAT exists as a dimeric protein in solution, there is one molecule of D-PhgAT in the asymmetric unit. This revealed that in the crystalline state each monomer of the dimeric enzyme adopts a similar conformation, unlike the asymmetric dimeric crystal structures of glutamine-1-semialdehyde aminomutase (Hennig *et al.*, 1997) and ornithine aminotransferase (Shen *et al.*, 1998). Assuming one monomer of 47.5 kDa D-PhgAT in the asymmetric unit, the calculated volume per unit mass, V_M (Matthews, 1968), is 2.46 Å^3 Da^{-1}, corresponding to a solvent content of 50.0%.

Preliminary phases for D-PhgAT have been obtained by a molecular-replacement technique using a modified GSA-AT structure. A full description of the procedure for obtaining phase information will be published elsewhere.

Table 1
Unit-cell parameters and data-collection statistics.

Space group	$P3_121$ or $P3_221$
Unit-cell parameters (Å)	$a = b = 75.155$, $c = 147.554$
Resolution (Å)	20–2.3
Temperature	100
Wavelength (Å)	0.9795
Total No. of reflections	487524
No. of unique reflections	22083
Data completeness	99.8
R_{sym}	0.065
Average $I/\sigma(I)$	8.7
Multiplicity	10.1
No. of molecules in asymmetric unit	1
V_M (Å^3 Da^{-1})	2.46
Solvent content (%)	50.0

Table 2
Statistics of data collection from a D-PhgAT crystal by resolution shell.

Resolution (Å)	No. of unique reflections	Completeness (%)	R_{sym}
7.27	753	95.1	0.040
5.14	1346	980	0.041
4.20	1688	990	0.040
3.64	1985	100	0.045
3.25	2218	100	0.056
2.97	2444	100	0.083
2.75	2668	100	0.112
2.57	2826	99.9	0.152
2.42	2960	99.9	0.203
2.30	3093	100	0.270

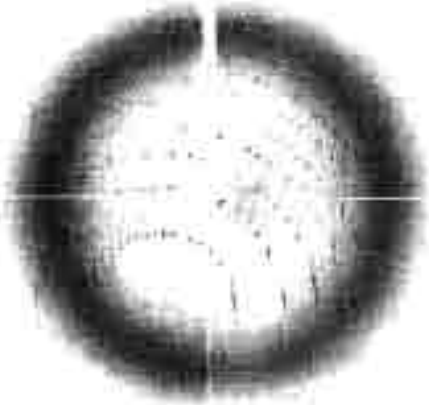

Figure 2
A typical 0.5° oscillation X-ray diffraction pattern of a D-PhgAT crystal.

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Appendix IV

Reprint

P. Kongsaeree, S. Prabpai, N. Sriubolmas, C. Vongvein, S. Wiyakrutta.
Antimalarial Dihydroisocoumarins Produced by *Geotrichum* sp.,
an Endophytic fungus of *Crassocephalum crepidioides*.
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Antimalarial Dihydroisocoumarins Produced by *Geotrichum* sp., an Endophytic Fungus of *Crassocephalum crepidioides*

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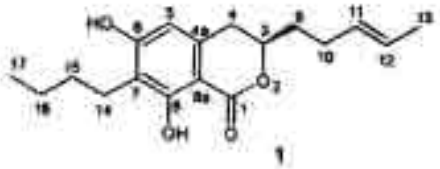
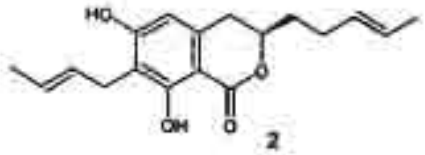
Three novel dihydroisocoumarin derivatives (**1**−**3**) with antimalarial, antituberculous, and antifungal activities have been isolated by bioassay-guided fractionation from an endophytic fungus, *Geotrichum* sp., collected from *Crassocephalum crepidioides*. Structures were established as 7-butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one (**1**), 7-but-15-enyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one (**2**), and 7-butyl-6,8-dihydroxy-3(*R*)-pentylisochroman-1-one (**3**) using spectroscopic data.

Approximately a quarter of all known biologically active natural products have been obtained from fungi, whose members are estimated to be around 1.5 million.[1−3] Endophytic fungi residing in the intercellular spaces of vascular plants are believed to be a fruitful source of biologically active secondary metabolites; however, they are relatively unexplored chemically.[4,5]

In our search for bioactive compounds from microorganisms, the crude extract of *Geotrichum* sp. (Endomycetaceae), an endophytic fungus isolated from *Crassocephalum crepidioides* S. Moore (Compositae), was found to be active against *Plasmodium falciparum* (K1, multi-drug-resistant strain) with an IC_{50} value of 0.63 μg/mL and against *Candida albicans* with an IC_{50} of 19.19 μg/mL. Bioassay-guided fractionation by Sephadex LH-20 and reversed-phase HPLC resulted in the isolation of three new dihydroisocoumarins (**1**−**3**).



1



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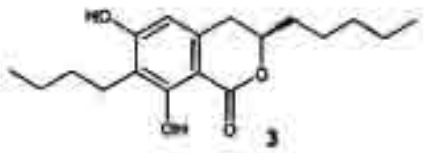

3

The crude EtOAc extract of fungal culture broth was passed through a Sephadex-LH20 column and was further purified by reversed-phase HPLC to obtain pure compounds, **1**−**3**. Compound **1** was isolated as a colorless solid, and its molecular formula was determined by accurate mass measurement (ESITOFMS) as $C_{18}H_{24}O_4$. In the ^{1}H NMR spectrum, the lone aromatic proton at δ 6.22 revealed a pentasubstituted aromatic ring, while the sharp singlet signal at δ 11.42 indicated the presence of an intramolecular hydrogen bond of a phenolic hydrogen to a carbonyl group. ^{13}C and DEPT NMR spectra revealed two methyl, six methylene, four methine, and six quaternary carbons. The ^{13}C carbonyl carbon signal at δ 170.5 indicated the presence of a lactone moiety whose carbonyl carbon was attached to the aromatic ring at C-8a (δ 138.2) and the lactone oxygen was connected to the C-3 methine carbon (δ 78.5). Correlations of the methylene protons (H-4, δ 2.84) to H-3 (δ 4.53) and the aromatic proton (H-5) to the C-4 methylene carbon (δ 32.9), respectively, in the COSY and HMBC experiments supported a dihydroisocoumarin skeleton. The COSY spectrum also revealed the presence of a butyl group and a *trans*-pentenyl group. The position of the butyl group was established to be at C-7 by the HMBC correlations of H-14 (δ 2.65) to C-7 (δ 114.8) and to two other aromatic carbons, with a hydroxyl group, at C-6 (δ 160.1) and C-8 (δ 162.2), respectively. A correlation of H-3 to H-9 (δ 1.94) in the COSY spectra as well as HMBC correlations of H-9 to C-3 and C-4 conclusively placed the pentenyl group at C-3 in the molecule. The doublet signal of the C-13 methyl group confirmed the position of the C-11−C-12 olefinic bond of the pentenyl moiety. The absolute configuration at C-3 was concluded to be *R* due to the fact that the circular dichroic spectrum of **1** showed a negative Cotton effect ascribed to the K-absorption band at 274 nm.[6−9] On the basis of the above observations, the structure of **1** was established as 3-(*R*)-7-butyl-6,8-dihydroxy-3-pent-11-enylisochroman-1-one.

Compound **2** had a molecular formula of $C_{18}H_{22}O_4$ as determined by ESITOFMS. ^{13}C and ^{1}H NMR spectra of **2** were very similar to those of **1** except for the marked differences in chemical shift values corresponding to C-15 and C-16. In the NMR spectra of **2**, the methylene signals of C-15 and C-16 were missing, and two additional olefinic protons (J = 15.6 Hz) were assigned to be at H-15 and H-16. Along with the doublet signal of the methyl group at C-17 and the COSY peak between H-17 and H-16, the NMR data led to the conclusion that **2** was 7-but-15-enyl-

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Table 1. ^{1}H and ^{13}C NMR Data (δ) for Compounds 1, 2, and 3 in $CDCl_3$[a]

	1		2		3	
no.	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1		170.5	-	170.3		170.4
3	4.53 m	78.5	4.54 m	78.5	4.50 m	79.2
4	2.84 m	32.9	2.84 m	32.9	2.81 m	32.9
4a		138.2	-	139.0		138.3
5	6.22 s	106.0	6.24 s	106.4	6.18 s	105.8
6		160.1		161.2		159.8
7		114.8		111.5		114.8
8		162.2		161.7		162.2
8a		101.8		101.7		101.8
9	1.94 m	34.5	1.95 m	34.5	1.86 m	34.7
10	2.22 m	27.7	2.21 br m	27.8	1.70 m	31.5
11	5.45 (15.2)	129.5	5.47 (15.2)	129.5	1.63 m	24.5
12	5.50 (15.2)	126.4	5.47 (15.2)	128.4	1.41 m	22.5
13	1.66 d (6.0)	17.9	1.67 d (6.0)	17.9	0.95 t (7.2)	14.0
14	2.85 t (7.6)	22.3	2.43 d (5.2)	25.7	2.83 t (7.5)	22.3
15	1.54 m	30.9	5.85 (15.6)	128.8	2.53 m	30.9
16	1.42 qn (7)	22.8	5.85 (15.6)	127.7	1.33 m	22.8
17	0.95 t (7.2)	14.0	1.72 d (5.6)	17.8	0.91 t (6.7)	14.0
8-OH	11.42 s		11.56 s		11.47 s	

[a] Coupling constants in parentheses.

Table 2. Bioactivities of Compounds 1, 2, and 3

compound	antimalarial[a] (IC_{50}, μg/mL)	anti-TB[b] (MIC, μg/mL)	antifungal[c] (IC_{50}, μg/mL)
1	4.7	25	19
2	inactive	50	inactive
3	2.6	inactive	33

[a] *Plasmodium falciparum* K1 (multi-drug-resistant strain). [b] *Mycobacterium tuberculosis* H37Ra. [c] *Candida albicans*.

6,8-dihydroxy-3-pent-11-enylisochroman-1-one. The structure of 2 was confirmed by the HMBC correlations between the doublet H-14 (δ 3.43, J 5.24 Hz) to the aromatic carbon at C-7 (δ 111.5) and the olefinic carbons C-15 and C-16.

The molecular formula of 3 was determined as $C_{18}H_{26}O_4$ from ESITOFMS data. ^{1}H and ^{13}C NMR data of 3 were analogous to those of 1 except that two additional methylene carbons replaced those of the olefin signals. The COSY spectrum between H_2-13 and H-12 and between H_2-9 and H-3, H-10 confirmed the position of the pentyl side chain at C-3 and thus led to the assignment of 7-butyl-6,8-dihydroxy-3-pentylisochroman-1-one for compound 3.

The configurations of dihydroisocoumarins 2 and 3 at C-3 were established by comparison of their optical rotation values with that of 1. Optical rotations measured for the three compounds were all negative. Hence, the configurations of 1–3 at C-3 were all proposed to be *R*.

The crude EtOAc extracts showed activity against *P. falciparum* K-1 as well as the fungus *C. albicans* and showed moderate potency against *Mycobacterium tuberculosis*. The activities of the purified individual dihydroisocoumarin (1–3) against the test organisms were compiled in Table 2.

Experimental Section

General Experimental Procedures. Melting points were determined using an Electrothermal melting point apparatus and were uncorrected. The ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were taken at the Central Instrumental Facility of Mahidol University on a Bruker DPX300 spectrometer, with TMS (δ_H 0 ppm) and $CDCl_3$ (δ_C 77.0 ppm) as internal references, respectively. HPLC was carried out on an HP1100 system using a Merck Lichrospher-C_{18} column (5 μm, 4.6 × 250 mm) with UV detection at 215 nm. Optical rotations were measured with a JASCO J-810 spectropolarimeter. IR spectra were recorded on an FT-IR system 2000 (Perkin-Elmer) spectrometer. TLC was performed on silica gel UV_{254} plates (Merck). ESITOFMS data were recorded on a Micromass LCT mass spectrometer.

Fungal Isolation. Fresh stems of *Crassocephalum crepidioides* S. Moore were collected from apparently healthy plants in the forest area of Songkhla Province, Southern Thailand. The stems were washed in running H_2O and surface sterilized by successive soaking in 70% EtOH for 1 min and sodium hypochlorite solution (5.25%) for 5 min and then rinsed twice with water. Aseptically, the stem rods were cut open and 2 × 5 mm^2 pieces of internal tissues were excised and placed on agar in Petri plates. After incubation at 25 °C, individual hyphal tips of the emerging fungi were removed and placed on potato dextrose agar (PDA). During the first two weeks of incubation, the cultures were periodically checked for purity and successively subcultured by hyphal tipping method until pure cultures were obtained. A fungal isolate, Ccre7, grew on PDA as a white filamentous fungus colony and produced arthroconidia upon maturation. The taxonomic identification of Ccre7 as *Geotrichum* sp. was performed based on fungal morphology and analysis of the DNA sequence of the ribosomal RNA gene region. Primers NS5 and ITS4 were used to amplified the ITS1–5.8S–ITS2 region from total DNA extracted from Ccre7.[10,11] The amplified DNA was purified and directly subjected to sequencing reactions using primers ITS2, ITS3, ITS4, and ITS5. BLAST was used to search for similar sequences in the GenBank. DNA sequences, analyzed by CLUSTALW and PAUP phylogenetic analysis programs, revealed that Ccre7 was a fungal member of the genus *Geotrichum*. The DNA sequence of ITS1–5.8S–ITS2 of Ccre7 has been submitted to GenBank (Accession Number AF481862). A subculture of the *Geotrichum* sp. Ccre7 has been deposited at the BIOTEC Culture Collection under the designation BCC 8964.

Bioassay Procedures. In vitro antimalarial activity was evaluated against the parasite *P. falciparum* (K1, multi-drug-resistant strain), which was cultured continuously according to the method of Trager and Jensen.[12] Quantitative assessment of antimalarial activity in vitro was determined by means of the microculture radioisotope technique based on the method described by Desjardins et al.[13] An IC_{50} value of 0.16 μg/mL (0.31 μM) was observed for the standard sample, chloroquine diphosphate, in the same test system. The antifungal activity was assessed against *C. albicans*, employing the colorimetric method.[14] In our system, the IC_{50} value of the standard drug, amphotericin B, was 0.01 μg/mL. Growth inhibitor activity against *Mycobacterium tuberculosis* H37Ra strain was determined using the Microplate Alamar Blue Assay (MABA).[15] The MIC values of the standard drugs isoniazid and kanamycin sulfate are 0.050 and 2.5 μg/mL, respectively, in our system.

Extraction and Isolation. *Geotrichum* sp. was grown on a PDA plate as an inoculum for 7 days at 25 °C. Six pieces of 5 × 5 mm blocks of the well-grown culture were inoculated into 200 mL of malt Czapek broth in a 1 L Erlenmeyer flask. The fungus was grown by still culture at 25 °C for 21 days. Fungal cultures (10 L) were filtered to remove mycelia and subsequently extracted twice with equal volumes of EtOAc. Evaporation of organic extracts in vacuo afforded 2.33 g of an oily orange-red residue. The crude extract was redissolved in methanol, loaded into a Sephadex LH-20 column (3 × 86 cm), and eluted by an isocratic system of MeOH. The eluate was collected in 15 mL fractions and combined to nine fractions on the basis of their TLC profiles. On the basis of bioactivity data, two fractions (6 and 7) were subsequently purified on a 4.6 × 250 mm Merck LiChrospher-C_{18} column with a gradient of MeCN–H_2O, 50:50 to 70:30, as the mobile phase with a flow rate of 1.0 mL/min to yield compound 1 (37 mg, t_R 20 min), compound 2 (10 mg, t_R 15 min), and compound 3 (6 mg, t_R 17 min).

7-Butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one (1): colorless solid (CH_2Cl_2); mp 158–160 °C; $[\alpha]^{25}_D$ −28.3° (c 0.25, MeOH); CD (c 3.42 × 10^{-4} M, MeOH) $\Delta\varepsilon$ (nm) 0 (291.3), −7.65 (274.3), 0 (256.8), 2.61 (249.5), 0 (242.7); UV (MeOH) λ_{max} (log ε) 220 (4.5), 274 (4.2) nm; IR ν_{max} (KBr) 3185, 2956,

1618, 1510, 1448, 1387, 1125 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 305.1756 [M + H]$^+$ (calcd for $C_{18}H_{25}O_4$, 305.1755).

7-But-2*S*-enyl-6,8-dihydroxy-3(*R*)-pent-1*E*-enylisochromman-1-one (2): colorless solid (CH_2Cl_2); mp 138–140 °C; $[\alpha]^{25}_D$ −28.0° (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 223 (4.1), 268 (3.7) nm; IR ν_{max} (KBr) 3150, 1618, 1503, 1449, 1384, 1266, 1198 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 303.1568 [M + H]$^+$ (calcd for $C_{18}H_{23}O_4$, 303.1597).

7-Butyl-6,8-dihydroxy-3(*R*)-pentylisochromman-1-one (3): colorless solid (CH_2Cl_2); mp 146–147 °C; $[\alpha]^{25}_D$ −20.0° (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 217 (4.6), 269 (4.2) nm; IR ν_{max} (KBr) 3188, 1618, 1503, 1444, 1389, 1307, 1126 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 307.1902 [M + H]$^+$ (calcd for $C_{18}H_{27}O_4$, 307.1901).

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