DISCLAIMER:

This document has been proofed and
its original formatting has been retained.

Active

Project #: G-33-643 Cost share #: Rev #: 2
Center # : 10/24-6-R7255-0A0 Center shr #: OCA file #:
Work type : RES
Contract#: 2 R01 HL34035-04A3 Mod #: BR DTD 3/2/93 Document : GRANT
Prime #: Contract entity: GTRC

Subprojects ? : Y CFDA:
Main project #: PE #: N/A

Project unit: CHEMISTRY Unit code: 02.010.136
Project director(s):
POWERS J C CHEMISTRY (404)894-4038

Sponsor/division names: DHHS/PHS/NIH / NATL INSTITUTES OF HEALTH
Sponsor/division codes: 108 / 001

Award period: 910701 to 920630 (performance) 920930 (reports)

Sponsor amount	New this change	Total to date
Contract value	0.00	198,548.00
Funded	0.00	198,548.00
Cost sharing amount		0.00

Does subcontracting plan apply ?: N

Title: SYNTHETIC ANTITHROMBOTIC AGENTS

PROJECT ADMINISTRATION DATA

OCA contact: Kathleen R. Ehlinger 894-4820

Sponsor technical contact	Sponsor issuing office
DR DIANE LUCAS (301)496-5911	MICHAEL MORSE (301)496-7257
DIVISION OF BLOOD DISEASES NATIONAL HEART, LUNG, & BLOOD INSTIT 9000 ROCKVILLE PIKE BETHESDA, MD 20892	GRANTS OPERATIONS BRANCH DIVISION OF EXTRAMURAL AFFAIRS NAT. HEART, LUNG, & BLOOD INTSTITUTE 9000 ROCKVILLE PIKE BETHESDA, MD 20892

Security class (U,C,S,TS) : U ONR resident rep. is ACO (Y/N): N
Defense priority rating : N/A NIH supplemental sheet
Equipment title vests with: Sponsor GIT X

Administrative comments -
ISSUED TO RETURN $1.44 IN UNEXPENDED FUNDS TO THE MAIN PROJECT FROM SUBPROJECT E-25-M85/KU.

GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

NOTICE OF PROJECT CLOSEOUT

Closeout Notice Date 03/24/93

Project No. G-33-643 Center No. 10/24-6-R7255-0A0

Project Director POWERS J C School/Lab CHEMISTRY

Sponsor DHHS/PHS/NIH/NATL INSTITUTES OF HEALTH

Contract/Grant No. 2 R01 HL34035-04A3 Contract Entity GTRC

Prime Contract No. _____

Title SYNTHETIC ANTITHROMBOTIC AGENTS

Effective Completion Date 920630 (Performance) 920930 (Reports)

Closeout Actions Required:	Y/N	Date Submitted
Final Invoice or Copy of Final Invoice	Y	
Final Report of Inventions and/or Subcontracts	Y	
Government Property Inventory & Related Certificate	N	
Classified Material Certificate	N	
Release and Assignment	N	
Other _____	N	

Comments CONTINUED BY G-33-E08. EFFECTIVE DATE 7-1-91.
CONTRACT VALUE $198,548.

Subproject Under Main Project No. _____

Continues Project No. G-33-676

Distribution Required:

Project Director	Y
Administrative Network Representative	Y
GTRI Accounting/Grants and Contracts	Y
Procurement/Supply Services	Y
Research Property Managment	Y
Research Security Services	N
Reports Coordinator (OCA)	Y
GTRC	Y
Project File	Y
Other HARRY VANN-FMD	Y
FRED CAIN-OOD	Y

NOTE: Final Patent Questionnaire sent to PDPI.
DHHS FORM 568 Required

GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

NOTICE OF PROJECT CLOSEOUT (SUBPROJECTS)

Closeout Notice Date 03/24/93

Project No. G-33-643 Center No. 10/24-6-R7255-0A0_

Project Director POWERS J C_____ School/Lab CHEMISTRY_____

Sponsor DHHS/PHS/NIH/NATL INSTITUTES OF HEALTH_____

Project # E-25-M85 PD KU D N Unit 02.010.126 T
GRANT # 2 R01 HL34035-04A3 MOD# BR DTD 3/2/93 MECH ENGR *
Ctr # 10/24-6-R-7355-0A2 Main proj # G-33-643 OCA CO KRE
Sponsor-DHHS/PHS/NIH /NATL INSTITUTES OF H 108/001
SYNTHETIC ANTITHROMB
Start 910701 End 920630 Funded 18,035.56 Contract 18,035.56

LEGEND
1. * indicates the project is a subproject.
2. I indicates the project is active and being updated.
3. A indicates the project is currently active.
4. T indicates the project has been terminated.
5. R indicates a terminated project that is being modified.

SECTION IV
PROGRESS REPORT SUMMARY

GRANT NUMBER
HL34035-05

PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR
Powers, James C.

PERIOD COVERED BY THIS REPORT
FROM 07/01/91 THROUGH 06/30/92

APPLICANT ORGANIZATION
Georgia Tech Research Corp.

TITLE OF PROJECT (Repeat title shown in item 1 on first page)
Synthetic Antithrombotic Agents

(SEE INSTRUCTIONS)

Specific Aims

1. Design and synthesize specific peptide-related reversible transition-state inhibitors for human thrombin.

2. Design and synthesize heterocyclic irreversible mechanism-based thrombin inhibitors.

3. Utilize molecular modeling and the x-ray structure of human thrombin to improve the potency and specificity of both peptide and heterocyclic inhibitors.

4. Evaluate the inhibitory potency and specificity of all new drugs *in vitro*.

5. Evaluate the *in vivo* efficacy of the antithrombotic agents in a rabbit thrombosis model.

The specific aims for the next budget period remain unchanged.

Progress Report

Summary. The majority of our efforts during the first year has been devoted to synthesis. The synthesis of the proposed inhibitors proved to be more difficult that we expected. Arginine related structures are much more time-consuming that derivatives related to other amino acids. However, only arginine derivatives are likely to be potent inhibitors of thrombin. We have not yet obtained new inhibitor structures in sufficient yields to undertake animal studies.

Specific Aim 1-Transition-state Inhibitors. We propose to synthesize arginine α-ketoesters RCO-Arg-CO-OEt by a Dakin-West reaction. The design of these transition state inhibitors is based on the interaction of 4-amidinophenylpyruvate (APPA) with trypsin. APPA is a potent competitive inhibitor of trypsin, thrombin, and factor Xa with K_I values of 1.6, 6.5 and 9.4 μM respectively. A refined x-ray crystal structure of the complex formed by bovine trypsin and 4-amidinophenylpyruvate has been determined. The amidinophenyl group is located in the primary specificity pocket (S_1) of trypsin in essentially the same location as the benzamidine ring in the benzamidine-trypsin complex, the active site serine of trypsin has added to the 2-carbonyl group in APPA to give a "tetrahedral" structure, and the oxyanion is interacting with the oxyanion hole of the protease. A unique feature of this structure is the hydrogen bonding observed between the carboxylate oxygen and the serine oxygen and the NH of histidine-57.

We have tried the Dakin-West reaction with a large variety of blocked arginine derivatives as listed in the following table, but have yet to obtain the arginine α-ketoester product in significant yields.

starting material [RCO-Arg(X)-OH]

RCO-	X
Z	H
Z	NO_2
Boc	H
Boc	NO_2
Boc	Z_2
Z-Leu	NO_2
Z-Val	NO_2
Boc-Val	Z_2

We have abandoned the synthetic route involving direct Dakin-West reactions on arginine derivative and will try to synthesize the arginine derivative indirectly. We next plan to try the Dakin-West reaction on ornithine derivatives. We have previously obtained good yields of α-ketoester products from lysine derivatives and thus ornithine would also be expect to react successfully. Once the ornithine derivative is prepared, we will convert the ornithine side chain into an arginine side chain by amidination with 3,5-dimethylpyrazole carboamidine. The synthetic routes which we will explore in the coming year are shown below.

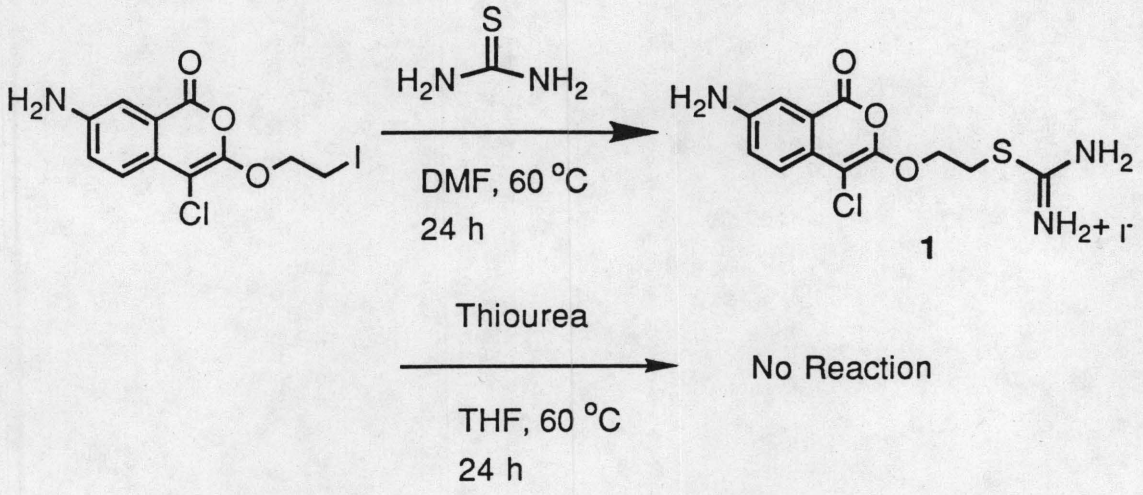
Z-Orn(Boc)-OH --> Z-Orn(Boc)-CO_2Et --> Z-Orn-CO_2Et --> Z-Arg-CO_2Et

Z-AA-Orn(Boc)-OH --> Z-AA-Orn(Boc)-CO_2Et --> Z-AA-Orn-CO_2Et -->

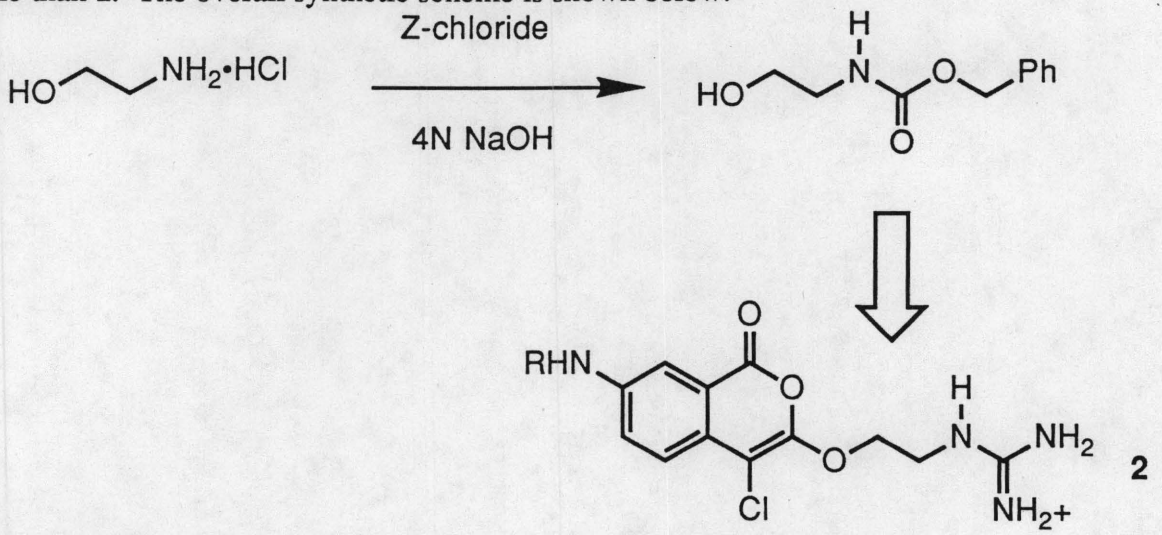
Z-AA-Arg-CO_2Et

Specific Aim 2-Mechanism-based Inhibitors. Isocoumarins containing basic substituents (aminoalkoxy, guanidino and isothiureidoalkoxy) in either the 3- or the 7-position are mechanism-based inhibitors for blood coagulation serine proteases and are anticoagulants in human plasma. Isocoumarins react initially with the active site Ser-195 to form an acyl enzyme which can deacylate to regenerate active enzyme. Alternately, the acyl enzyme can eliminate chlorine to form a quinone imine methide intermediate which can react either with a nearby enzyme nucleophile such as His-57 to give an alkylated enzyme or with water (or another solvent nucleophile). We propose to synthesize a number of isocoumarins with varying substituents of the 7-amino group to provide specificity for thrombin and other coagulation enzymes.

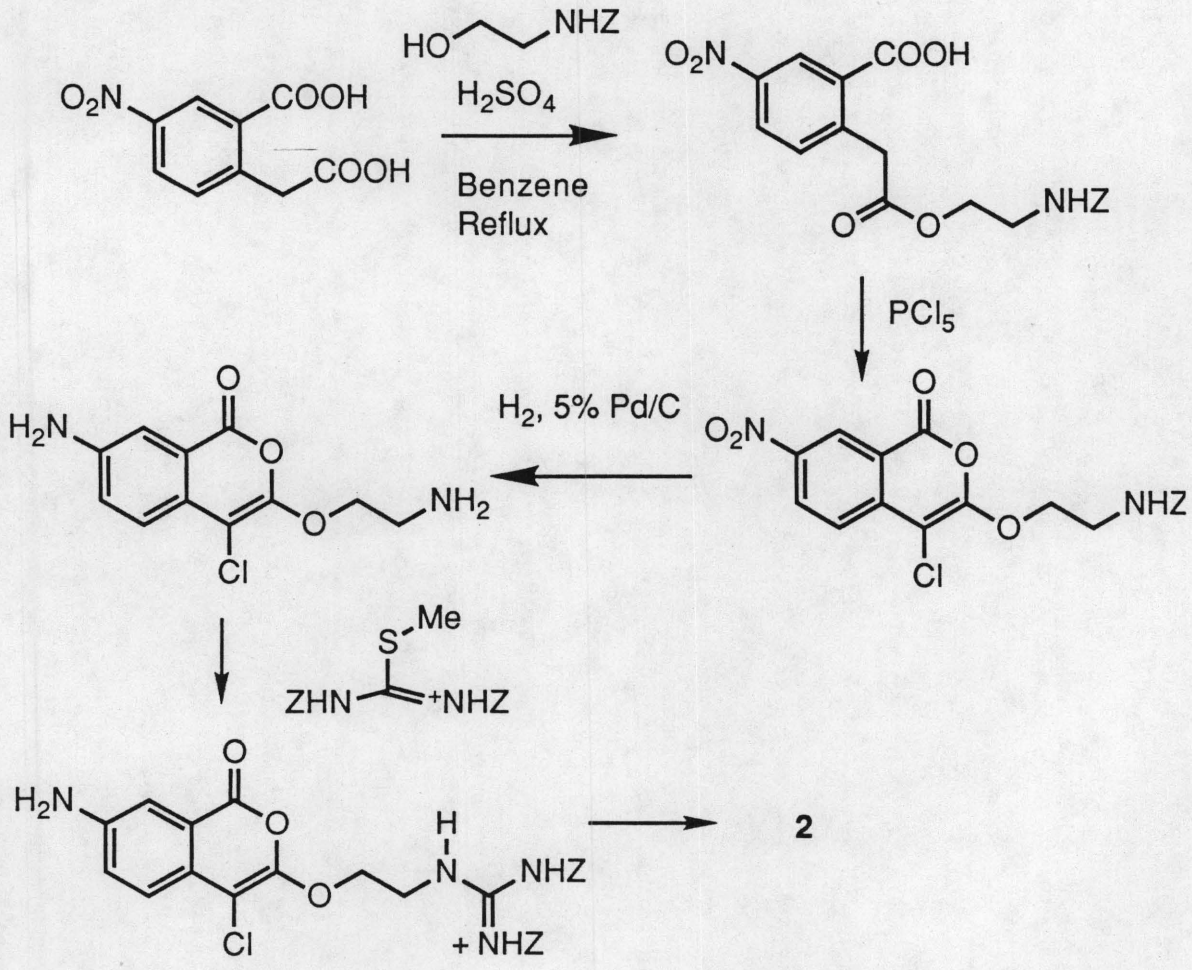
One of our major targets is compound **1** which is an analog of ACITIC, which we have previously studied in animals. This isocoumarin is projected to be more reactive than ACITIC. We have obtained the product in low yields by the route shown below, but unfortunately considerable decomposition is observed. Milder conditions do not results in production of the product.



We plan to synthesize an alternate structure **2** in the coming year. This compound may be easier to synthesize than **1**. The overall synthetic scheme is shown below.



A detail proposed synthetic pathway is shown below.



Specific Aim 3-X-ray Structural Studies. Dr. Wolfram Bode at the Max Planck Institute for Biochemistry in Munich, West Germany is trying to obtain crystals of bovine or human thrombin inhibited by three of our isocoumarins for x-ray crystallographic studies. Thus far, they have been unable to obtain suitable crystals. Dr. Bode plans to continue efforts aimed at obtaining a crystalline derivative. In addition, Jay Bertrand a graduate student in the research group of Dr. Bud Suddath at Georgia Tech is carrying out x-ray studies with trypsin inhibited by several of our isocoumarin inhibitors. He has crystals of several derivatives and is collecting x-ray diffraction data. The work has gone slowly due to instrument breakdowns. However, all the background work has been accomplished and it is likely that we will have an isocoumarin x-ray structure with trypsin in the next few months. We will then use it for modeling in the active site of thrombin.

Specific Aim 4-In Vitro Studies. We have been carrying out kinetich studies aimed at preparing chemically coupled hybrids of Fab fragments and synthetic thrombin inhibitors. We plan to construct hybrid molecules of synthetic thrombin inhibitors coupled to Fab fragments which are directed against fibrin and platelets. Specifically, we will prepare and test a hybrid with a Fab molecule coupled to one D-FPR-CH_2Cl (an irreversible thrombin inhibitor). The Fab fragments directed against fibrin and platelets will be prepared in the laboratory of Marshall Runge at Emory University and will contain one reactive thiol group in their structures.

We have synthesized two double-headed derivatives of D-FPR-CH_2Cl (D-Phe-Pro-Arg-CH_2Cl) and worked out the procedure for coupling these derivatives to the thiol group of proteins using albumin as a model system. The two double headed derivatives are $ClCH_2$-Arg<-Pro<-D-Phe-CO-CO-D-Phe-Pro-Arg-CH_2Cl and $ClCH_2$-Arg<-Pro<-D-Phe<-CO-$(CH_2)_3$-CO-D-Phe-Pro-Arg-CH_2Cl (<- indicates a reversed peptide chain). The first inhibitor was prepared by reacting D-FPR(Tos)-CH_2Cl with oxalyl chloride to give [-CO-D-FPR(Tos]-$CH_2Cl]_2$ which was then deblocked with anhydrous HF and purified on SE-Sephadex. The second double headed chloromethyl ketone molecule $ClCH_2$-Arg<-Pro<-D-Phe<-CO-$(CH_2)_3$-CO-D-Phe-Pro-Arg-CH_2Cl was synthesized similarly by reacting D-FPR(Tos)-CH_2Cl with glutaryl dichloride followed by deblocking. Both double headed molecules are potent irreversible thrombin inhibitors, although neither is as reactive as the parent D-FPR-CH_2Cl. The synthetic work was carried out prior to the initiation of this grant by a postdoc supported by an industrial training grant.

Coupling of each inhibitor to the thiol group of albumin was carried out by reacting an excess of the double headed inhibitor with albumin in a 0.1 M $NaHCO_3$, pH 8.1 buffer. By thiol titration, we found that respectively 45% and 40% of the thiol groups in albumin reacted with the double headed D-FPR-CH_2Cl inhibitors to give adduct I and adduct II. Both adducts I and II inhibit thrombin quite potently with second order inhibition rates of 29,000 and 78,000 $M^{-1}s^{-1}$ respectively. Adduct II has the structure albumin-S-CH_2-Arg<-Pro<-D-Phe<-$CO(CH_2)_3$-CO-D-Phe-Pro-Arg-CH_2Cl where a tripeptide-glutaryl spacer links one reactive D-FPR-CH_2Cl molecule to the thiol group of albumin. This spacer is more flexible than the one in adduct I and this is likely the reason for the 3 fold higher inhibition rate with thrombin by adduct II. Adduct II is very stable in 0.1 M Hepes, pH 7.5 buffer and has a half-life of 3 days. These experiment clearly demonstrate that we can covalent link synthetic thrombin inhibitors to thiol groups in proteins and produce stable protein adducts which contain potent thrombin inhibitors. We propose to use these same reactions to prepare adducts of antiplatelet and antifibrin Fab fragements with both double headed thrombin inhibitors. We have now obtained the Fab fragment and will carry out these experiments in the near future.

Specific Aim 5-Vertebrate Animal Studies. Since none of the synthetic work has yet been completed, no animal work was carried out during the first year of this research. We plan to carry out the animal studies originally proposed as soon as inhibitor molecules are available.

Publications

Synthetic Substrates And Inhibitors For Serine Proteases From Lymphocytes, Mast Cells, And Blood, Powers, J. C., Odake, S., Ueda, T., Hudig, D., Graves, H., and Kam, C.-M. (1992) in *Toward Understanding the Molecular Basis of Kinin Action, Kinin 1991 International Conference*, in press.

Synthetic Substrates and Inhibitors of Thrombin, Powers, J. C., and Kam, C-M. (1992) in *Thrombin: Structure and Function* (Berliner, L. J., Ed.) in press, Plenum Publishing Corp., New York.

Program Income

None