

ture analysis program system UNICS^{3,27} Further results of the crystallographic experiment are available and are described in the supplemental material paragraph.

Molecular Mechanics Calculation. All the plausible initial geometries of **6** were generated automatically with use of our program (MMRS) using 30° of dihedral angle resolution. The closure bond was chosen to be the bond between C₂ and C₃. The constraints of the closure bond distance and angle were chosen to be in the range of 1.0–2.3 Å and 90–130°, respectively. The resulting 28 geometries that satisfy the ring closure criteria were then minimized with Allinger's MM2 force field using the following optional parameters for the benzene sp² carbon:

torsion	V1	V2	V3
1-2-2-2	-0.270	9.950	0.0
1-2-2-5	0.0	9.950	0.0
2-2-2-2	-0.930	9.950	0.0
2-2-2-5	0.0	9.950	-1.06

stretching (2-2) 8.07 (ks) 1.3937 Å (Lo)

Ten initial geometries out of 28 initial sets converged to one of the three syn conformers. The steric energies of the three syn conformers

are 9.26 (syn boat-boat), 9.28 (syn chair-chair), and 9.47 kcal/mol (syn chair-boat). The 18 remaining initial geometries gave several, so-called anti structures. The steric energy of the lowest one of these anti forms was 15.95 kcal/mol.

Variable-Temperature NMR Spectra. A sample of the hydrocarbon **6** was dissolved in a mixture of CS₂-CD₂Cl₂-CDCl₃ (1:1:1). Spectra (¹H) were recorded on a JEOL GX-400 and are reproduced in Figures 5 and 6. Variable-temperature ¹³C NMR spectra (100 MHz) were measured in a mixture of CS₂-CD₂Cl₂ (2:3). A spectra width of 20 000 Hz, a filter bandwidth of 10 000 Hz, an acquisition time of 1.25 s, a pulse width of 4.5 s, and 16 000 data points with 1.5-s pulse delay were used.

Registry No. **6**, 116633-68-2; **7**, 109757-55-3; **8**, 109757-64-4; (*m*-bromophenyl)acetonitrile, 31938-07-5; 2-(*m*-bromophenyl)-2-methylpropionitrile, 90433-20-8.

Supplementary Material Available: Tables of the final atomic coordinates, isotropic and anisotropic thermal parameters, bond distances, and bond angles and variable-temperature ¹³C NMR spectra for **6** (7 pages). Ordering information is given on any current masthead page.

Solid-Phase Synthesis of Porcine Cardiodilatin 88[†]

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Contribution from the Anatomisches Institut der Universitaet Heidelberg, D-6900 Heidelberg, FRG, and Applied Biosystems Japan Inc., Edogawa-ku Tokyo 136, Japan. Received April 4, 1988

Abstract: An 88 amino acid residue peptide, porcine cardiodilatin pCDD 88, first isolated from porcine atria by a brain-gut hormone isolation protocol, was synthesized on an improved solid-phase peptide synthesizer and then purified. The homogeneity and primary structure were confirmed by several criteria, including two types of immunoassay. The synthetic material confirmed the proposed primary structure of natural pCDD 88. The results also illustrate the utility of solid-phase automated peptide synthesis using the *t*-Boc strategy in combination with HF cleavage for the preparation of relatively large peptides (*M_r*, ca. 9400).

By functional and morphological methods it was revealed that mammalian atrial myocytes contain biologically active peptides stored in specific secretory granules.¹ Recently, several structurally related peptides have been isolated from mammalian atria and characterized. At the same time, the precursor of the atrial peptide of several species was deduced from cDNA analysis.² These peptides were termed collectively atrial natriuretic factor (ANF) or atrial natriuretic peptide (ANP), which have potent natriuretic, diuretic, and vasorelaxant activities. There is now agreement that atrial myocyte granules contain the pre-hormone consisting of 126 amino acid residues and that its C-terminal fragment is a circulating form.² However, very little is known about the precursor itself as well as the biosynthetic pathways to these molecules.

Cardiodilatin (CDDs) were first isolated from porcine atria and characterized as atrial peptides by Forssmann et al.^{3,4} The amino acid sequence of pCDD is similar to that of human τ -ANP.⁵ The primary structures of porcine and human pro-atrial peptides are shown in Figure 1. Besides pCDD 126, pCDD 88 was also isolated from porcine atria in significant amounts⁴ according to the isolation strategy for gastrointestinal hormones.⁶ In this

method, peptides in crude extracts were adsorbed on alginic acid and ethanol precipitation was employed as further purification. The primary structure of pCDD 88 is identical with the positions 39–126 of pCDD 126 (Figure 1), and it is believed⁷ that pCDD 88 is generated intact in the biosynthetic pathways. On the other hand, immunoassays in combination with high-performance liquid chromatography (HPLC) showed that pCDD 88 might be an artifact of the isolation and purification procedure.⁸

Biologically active peptides are now possible to synthesize with an improved solid-phase procedure using high-quality reagents. The purpose of this study is to test the feasibility of synthesizing an 88 amino acid residue peptide by using an automated syn-

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[†] A part of this study was presented at the 24th Symposium on Peptide Chemistry, Tokyo, Japan, October 1986. All amino acids except glycine are of the L configuration.

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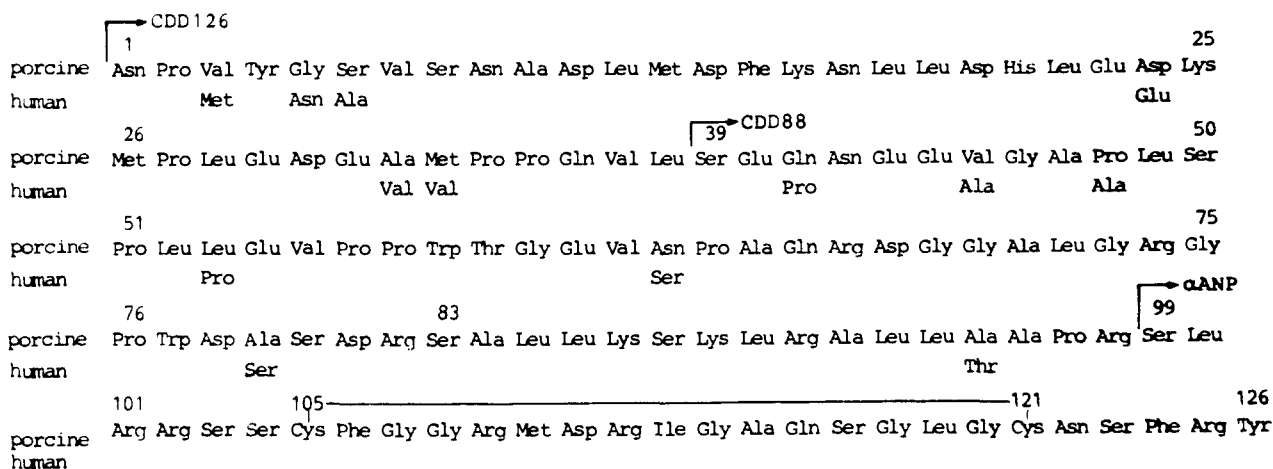


Figure 1. Primary structure of cardiostatins.

thesizer and to determine its authenticity by biological and immunochemical assays. During large-chain assembly in a complex peptide synthesis, the prevention of formation of deletion, termination, and modification peptides is paramount to the success of the synthesis. From this point of view, the synthesis of pCDD 88 was performed semiautomatically using an automated peptide synthesizer of Applied Biosystems Inc. (ABI Model 430A) with modified programs utilizing the t-Boc strategy with benzyl-type side-chain protection and phenylacetamidomethyl-derivatized polystyrene support (PAM-resin).⁹ The fidelity of the chain assembly was assessed by quantitative ninhydrin monitoring¹⁰ of each amino acid coupling in the C-terminal region. In addition, automated Edman degradation was carried out at several positions with the ABI-470A gas-phase sequencer in combination with online PTH analysis (ABI-120A) to confirm the correct amino acid sequence of the resin-bound peptides.¹¹ The synthesis was divided into three stages. The first was the synthesis of the C-terminal 28-residue peptide; pCDD(99-126), identical with to α -hANP.⁵ This synthesis was repeated on three separate occasions to find optimized recoupling conditions. The final synthesis used repetitive recoupling to gain optimal yield as judged by quantitative ninhydrin monitoring.¹⁰ After incorporation of the Ser residue at position 99, a part of the peptidyl resin was cleaved and deprotected to provide α -hANP with satisfactory purity. The second part was the synthesis of the 44-residue peptide pCDD(83-126). At an intermediate stage, cleavage of an aliquot of the peptidyl resin gave the C-terminal 44-residue peptide which was used to confirm the correct chain assembly. This material served as a useful substrate for processing to the C-terminal fragment (α -hANP), which is probably generated by enzymatic cleavage of the Arg-Ser bond. The final stage of the synthesis was further elongation of the pCDD(83-126) fragment to residue 39.

Peptide-Chain Assembly, Cleavage, and Purification of pCDD(99-126)/ α -hANP. The synthesis of pCDD(99-126) is summarized in Table I. The incorporation of Boc-amino acid derivative was performed by using double-, triple-, or quadruple-coupling cycles to gain optimal yield as estimated by ninhydrin monitoring.¹⁰ The residues, at positions 112, 109, 104, and 103, were incorporated by repeated coupling. Although the quantitative ninhydrin test appeared to show less than 99% incorporation of these residues, actual incorporation as determined by preview sequence analysis¹¹ showed greater than 99% efficiency. After incorporation of Ser⁹⁹, an aliquot of peptidyl resin was back-sequenced to show the desired amino acid incorporation up to position 125, while position 126 (Tyr) was linked to the solid

Table I. Solid-Phase Synthesis of pCDD(99-126)/ α -hANP

	position	side-chain protectn	coupling no.	yield, ^a %
126	Tyr-PAM-resin	BrZ	-	(0.50 mmol)
	Arg	Tos	+	99.9
	Phe		+	99.5
	Ser	BzL	+	99.4
	Asn		++	99.6
	Cys	MBzl	+	99.8
120	Gly		+	99.6
	Leu		+	99.5
	Gly		++	99.6
	Ser	Bzl	++	99.8
	Gln		++++	98.7
	Ala		++	99.5
	Gly		++	99.5
	Ile		++	98.9
	Arg	Tos	++++	95.2 ^b
	Asp	OBzl	++	99.0
110	Met		++	99.4
	Arg	Tos	++++	93.3 ^b
	Gly		+++	98.9
	Gly		++	99.3
	Phe		+++	99.3
	Cys	MBzl	+++	99.3
	Ser	Bzl	+++	92.8 ^b
	Ser	Bzl	++	99.3
100	Arg	Tos	++++	97.8 ^b
	Arg	Tos	++++	ND
	Leu		+++	ND
99	Ser	Bzl	+++	ND

^aQuantitative ninhydrin test. ^bOnline sequencing >99%.

support. Successive runs starting from Boc-Tyr(2-BrZ)-PAM resin (0.5 mmol) provided peptidyl resins corresponding to pCDD(99-126) (1.95-2.00 g). Relatively significant material loss was observed through autosampling. A part of the peptidyl resin was treated with trifluoroacetic acid (TFA) to remove the N-terminal Boc group and cleaved with HF in the presence of cation scavengers. The resulting deprotected peptides were extracted with aqueous acetic acid and oxidized with potassium ferricyanate. Completion of disulfide bond formation was determined according to Ellman.¹² Half of the crude oxidized product was desalted by gel filtration on Sephadex G25. The peptide fractions were pooled and submitted to gel filtration on Sephadex G50. The major peak fractions 53-69 and the minor peak fractions 41-52 [relative molecular mass (M_r) ca. 3000 and 6000, respectively, on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Swank and Munkres¹³] were pooled and lyophilized. Ultrafiltration, using Amicon YC05

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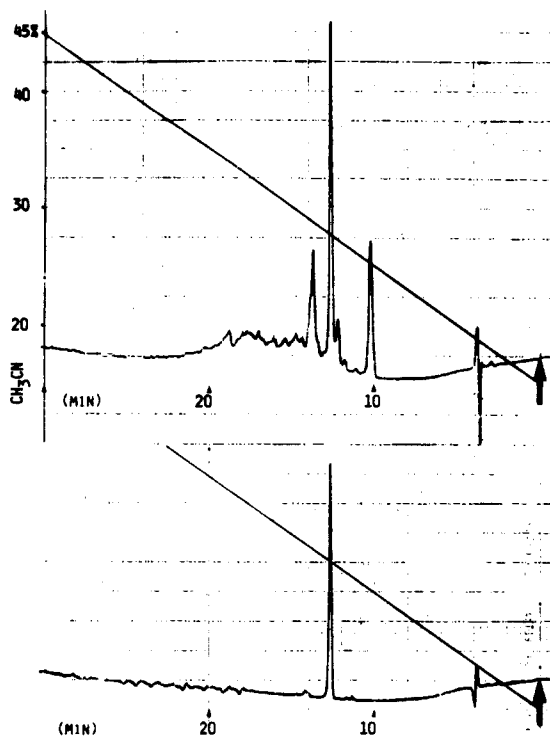


Figure 2. Elution profiles of synthetic pCDD(99-126) in HPLC. Top: crude material after gel filtration. Bottom: purified material. Column, TSK-GEL ODS 120T 4.6 × 250 mm; eluent, 0.01 M HCl/CH₃CN, 85/15-55/45 (v/v) in 30 min; flow rate, 1.0 mL/min; absorbance, 210 nm.

Table II. Amino Acid Compositions of pCDD-Related Peptides^a

amino acid	peptide ^b			
	1	2	3	4
Ala	1.1 (1)	4.8 (5)	9.2 (9)	8.6 (9)
Arg	4.7 (5)	6.3 (7)	10.1 (10)	9.1 (10)
Asx	1.8 (2)	1.7 (2)	7.0 (7)	7.0 (7)
Cys	2.0 (2)	1.7 (2)	2.0 (2)	1.6 (2)
Glx	1.0 (1)	1.0 (1)	7.1 (8)	8.6 (8)
Gly	4.9 (5)	5.0 (5)	11.5 (11)	9.5 (11)
Ile	0.9 (1)	1.0 (1)	0.9 (1)	1.0 (1)
Leu	2.0 (2)	6.5 (7)	10.8 (11)	9.8 (11)
Lys		2.3 (2)	2.6 (2)	4.8 (2)
Pro		1.3 (1)	6.7 (7)	6.6 (7)
Phe	2.1 (2)	2.1 (2)	2.4 (2)	2.2 (2)
Met	1.0 (1)	1.1 (1)	1.2 (1)	1.3 (1)
Ser	5.2 (5)	7.2 (7)	10.8 (10)	7.9 (10)
Thr			0.9 (1)	1.0 (1)
Trp			ND (2)	ND (2)
Tyr	1.1 (1)	1.0 (1)	1.3 (1)	1.0 (1)
Val			2.5 (3)	3.3 (3)

^aPico-Tag method: 6 N HCl, 110 °C, 12 h. ^bPeptides: (1) synthetic pCDD(99-126), (2) synthetic pCDD(83-126), (3) synthetic pCDD 88/pCDD(39-126), (4) natural pCDD 88.

membrane (M_r cutoff 500), of the other half of the oxidation mixture gave after concentration and desalting slightly higher purity material as determined by HPLC analysis. Both materials were easily purified by one-step reverse-phase HPLC (Figure 2). The purified material showed retention on HPLC identical with the material obtained by conventional solution synthesis.¹⁴ Amino acid ratios by the Pico-Tag method gave satisfactory values (Table II, column 1). Sequence analysis as well as tryptic mapping showed the predicted primary structure. This purified material showed strong relaxant activity¹⁵ on rabbit aorta. Thus, the chain

Table III. Solid-Phase Synthesis of pCDD(83-126)

	position	side-chain protectn	yield, ^a %
	Protected α -hANP-resin (1.95 g)		
98	Arg	Tos	93.2
	Pro		98.2
	Ala		99.6
	Ala		99.0
	Leu		99.0
	Leu		99.0
	Ala		98.8
90	Arg	Tos	95.4
	Leu		98.4
	Lys	ClZ	98.8
	Ser	Bzl	99.1
	Lys	ClZ	98.5
	Leu		98.3
	Leu		98.1
83	Ala		99.1
	Ser	Bzl	98.1
			(1.73 g)

^aUncorrected value.

assembly to position 99 was judged to be successful.

Peptide-Chain Assembly and Purification for pCDD(83-126). Starting from pCDD(99-126)-resin, double-coupling cycles and capping protocols using acetic anhydride as the acylation reagent were employed. The method was based on the Applied Biosystems VERSION 1.20 double-coupling cycles with a few modifications as summarized in Figure 3. The synthesis is shown in Table III, in which the coupling yields were uncorrected for capping. An aliquot of the resulting pCDD(83-126)-resin was sequenced to confirm the correct chain assembly. A part of this resin was cleaved with HF containing methyl ethyl sulfide and anisole as scavengers, washed with absolute ether, extracted with aqueous AcOH, and lyophilized. The lyophilizate was reduced with dithiothreitol and desalted on Sephadex G25. The major peptide fractions were pooled, diluted, and oxidized in air. Sulfhydryl content of the peptide was determined according to Ellman.¹² After gel filtration of the oxidized material on Sephadex G25, the major peptide component was purified on HPLC (Figure 4). The primary structure of the purified material was confirmed by gas-phase sequencing and amino acid analysis (Table II, column 2) as well as tryptic digestion. This material showed a strong relaxant activity on rabbit aorta in a dose-dependent manner (Figure 5).

Peptide-Chain Assembly, Cleavage, and Purification for pCDD 88. With the same protocol as illustrated in Figure 3, further peptide-chain assembly is summarized in Table IV. In addition to the side-chain protecting groups mentioned before, the cyclohexyl group (OcHex)¹⁶ was used for the β -carboxyl function of Asp (positions 68 and 78) to minimize aspartimide formation during assembly and cleavage, and the formyl (CHO) group was used for indole protection of Trp.^{17a,b} The problematic Pro⁵⁶ Pro⁵⁷ was coupled as a dipeptide Boc-Pro-Pro-OH.¹⁸ After this dipeptide was recoupled, its actual incorporation was confirmed by sequencing to position 79. Further chain assembly to pCDD 88-resin was performed without additional modification to the general protocol as outlined above. The N^α -Boc group of the peptidyl resin was removed on the synthesizer and the N^α -deprotected resin was treated with HF by the low-high method.¹⁹ After being washed with absolute ether to remove residual scavengers, the peptide was extracted with aqueous AcOH and lyophilized. The resultant material was gently oxidized in an open

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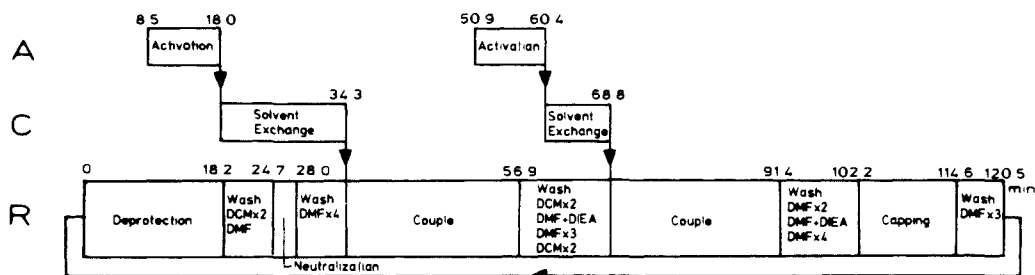


Figure 3. Schematic diagram of a typical double-coupling and capping cycle showing parallel overlapping of operation of the activator (A), concentrator (C), and reaction vessel (R). DCM, dichloromethane; DMF, *N,N*-dimethylformamide; DIEA, *N,N*-diisopropylethylamine.

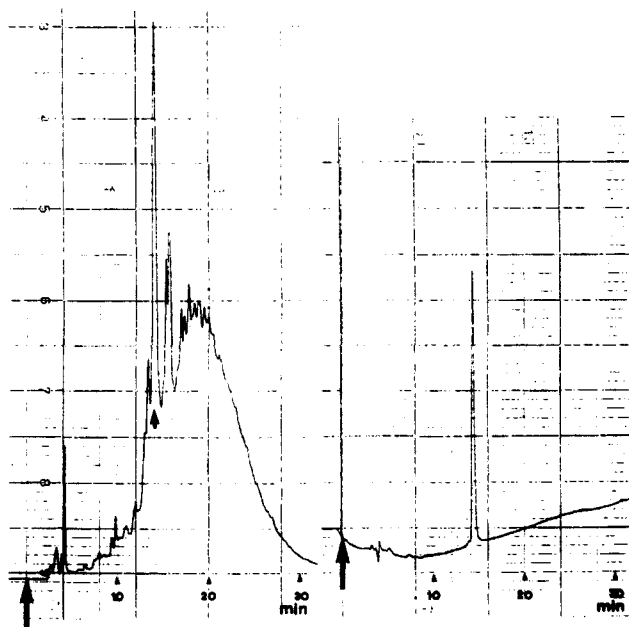


Figure 4. Elution profiles of synthetic pCDD(83-126) in HPLC. Left: material after gel filtration. Right: purified material. Column, TSK-GEL ODS 120T 4.6 × 250 mm; eluent, 0.01 M HCl/CH₃CN, 80/20-60/40 (v/v) in 30 min; flow rate, 1.0 mL/min; absorbance, 210 nm.

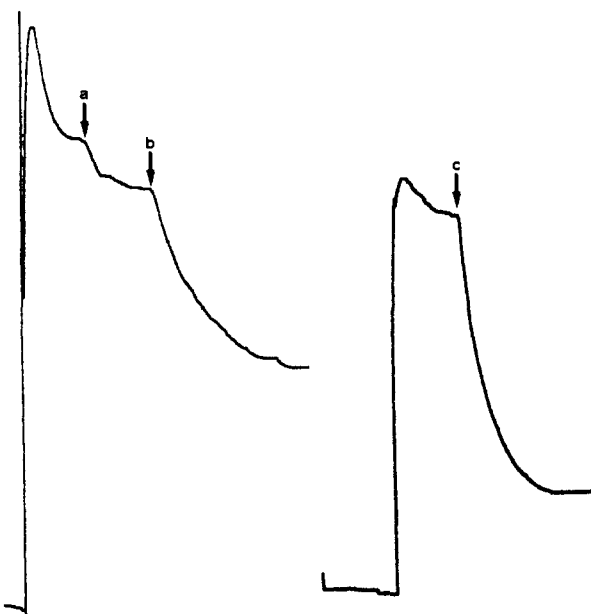


Figure 5. Rabbit aorta relaxant activity of purified synthetic pCDD(83-126). Key: a, 5 ng; b, 25 ng; c, 100 ng.

vessel at a peptide concentration of 0.1–0.2 mg/mL at room temperature for 3 days. Sulfhydryl content was monitored as before. Salts and small peptides were removed by repeated dialysis using Spectra/Por 6 membrane (M_r cutoff 2000). The dialysate

Table IV. Solid-Phase Synthesis of pCDD 88

position	side-chain protectn	yield, ^a %
Protected pCDD(83-126)-resin (1.14 g)		
82	Arg Tos	
	Asp OBzl	
80	Ser Bzl	
	Ala	
	Asp OcHex	
	Trp CHO	98.9
	Pro	97.9
	Gly	98.5
	Arg Tos	97.6
	Gly	98.6
	Leu	
	Ala	
70	Gly	
	Gly	
	Asp OcHex	
	Arg Tos	
	Gln	
	Ala	
	Pro	
	Asn	
	Val	
	Glu OBzl	
60	Gly	
	Thr Bzl	
	Trp CHO	
57	Pro	
56	Pro Boc-Pro-Pro-OH	
	Val	
	Glu OBzl	
	Leu	
	Leu	
	Pro	
50	Ser Bzl	
	Leu	
	Pro	
	Ala	
	Gly	
	Val	
	Glu OBzl	
	Glu OBzl	
	Asn	
	Gln	
40	Glu OBzl	
39	Ser Bzl	

(1.10 g)

^a Corrected value.

was lyophilized and submitted to gel filtration in a 2.1 × 115 cm column of Sephadex G50 in 1 M AcOH. The fractions 35–45 were pooled and lyophilized to afford partially purified pCDD 88. An HPLC profile of this material is shown in Figure 6 (left) and had an apparent M_r of 9000 of SDS-PAGE¹³ in nonreducing conditions. This is consistent with the M_r of 9411 calculated from the proposed sequence.⁴ In addition, the authenticity of the resulting material was confirmed by Western blots using antibodies HD16 (specific to α -hANP) and NA2F12 (specific to pCDD 88).⁸ Further purification of the partially purified pCDD 88 was carried out by cation-exchange chromatography on carboxymethyl cel-

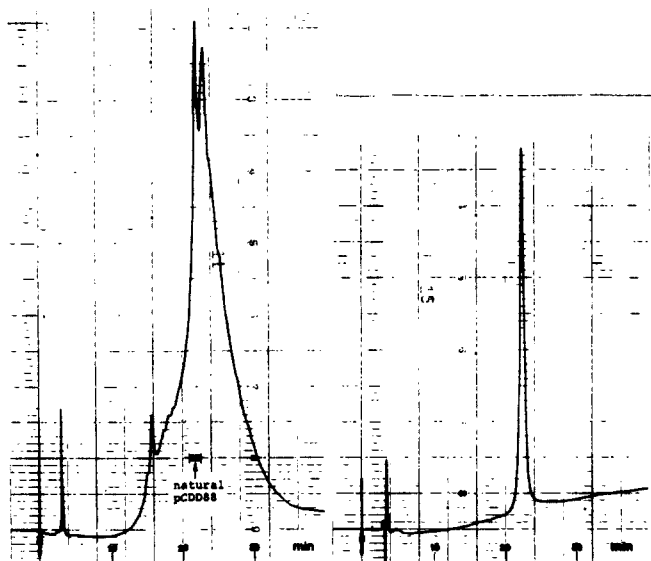


Figure 6. Elution profiles of synthetic pCDD 88 in HPLC. Left: partially purified pCDD 88. Right: purified synthetic pCDD 88. Column, Senshu Pak VP318-1251 4.6 × 250 mm; eluent, 0.01 M HCl/CH₃CN, 82/20–60/40 (v/v) in 40 min; flow rate, 1.0 mL/min; absorbance, 210 nm.

lulose or by chromatofocusing but provided no significant effect or indeed practical yield. In addition, immunoaffinity chromatography using monoclonal antibody NA2F12⁸ as ligand was employed for purification of the above partially purified pCDD 88 followed by Sephadex G75 chromatography to remove affinity ligand and salts. Finally, purified material was obtained by reverse-phase HPLC. The purified synthetic pCDD 88 was coeluted with the natural pCDD 88 as a single sharp peak on HPLC in the elution system illustrated in Figure 7. In addition, gas-phase sequencing gave the desired primary structure and amino acid analysis gave satisfactory results (Table II, column 3). Furthermore, tryptic digests were submitted to HPLC (Figure 8), and each peak was collected and subjected to gas-phase sequencing and amino acid analysis. All peaks were identified as the predicted fragments (Figure 9).

Biological and Immunochemical Properties of Synthetic pCDD 88. The *in vitro* bioassay of synthetic pCDD 88 was carried out using rabbit aorta smooth muscle strips. The synthetic pCDD 88 showed equipotent relaxant activity as a natural pCDD 88 (Figure 10). However, this activity seems to be related to the C-terminal segment involving S–S linkage between positions 105 and 121 of the precursor. Moreover, the synthetic fragments in the N-terminal region such as CDD(8–24), CDD(40–55), and CDD(80–94)²⁰ showed no activity in this assay system.²¹ Preliminary *in vivo* experiments with the synthetic pCDD(99–126), pCD(83–126), and pCDD 88 showed significant hypotension, natriuresis, and diuresis in conscious dogs. The details of these physiological experiments will be published elsewhere.

Immunochemical characterization was carried out with two types of immunoassay system. First, radioimmunoassay (RIA) of α -hANP, which is widely used to assay atrial hormone, was carried out. Dose–response curves of both natural and synthetic pCDD 88 are shown in Figure 11. Second, we have developed a two-site sandwich enzyme-linked immunoadsorbent assay (ELISA) system for CDD 88 with the use of purified polyclonal antibody NAHD-16 in combination with monoclonal antibody NA2F12.⁸ The serial dilution curve of synthetic pCDD 88 in the above ELSIA showed that the synthetic material was identical with natural material in this system (Figure 12). Thus, the purified synthetic pCDD 88 behaved in the same manner as the natural one in both the RIA and ELISA systems.

The synthetic pCDD 88 was shown to be identical with the natural one as far as it was examined. Collectively, these data indicate that the synthetic products contain a high proportion of molecules with the correct chemical structure and with the expected biological and immunochemical properties. At the same time, the results confirmed the proposed structure of the natural material.

The significance of the precursor to atrial hormone itself as well as N-terminal segments to the precursor is still unknown. Several multihormone-producing precursors are known such as opioid peptide precursors²² and the VIP-PHI precursor,²³ and it might be expected that pro-atrial peptide could generate more than one biologically or physiologically active peptide fragment through endogenous enzymatic processing. The biological and physiological significance of the precursor-related peptides such as pCDD(83–126), pCDD 88, or any N-terminal segments, for example, pCDD(1–38) or pCDD(1–98), remains to be clarified. The synthetic peptides prepared in this study provide a tool for investigating the physiological significance as well as the biosynthetic pathway of atrial hormones.

Experimental Section

The protected amino acid derivatives except Boc-Asp(OcHx)-OH were obtained in 2.0-mmol quantities in prepacked cartridges from ABI. Boc-Asp(OcHx)-OH was purchased from Peptide Institute Inc. and used in 2.0-mmol quantities. Boc-Pro-Pro-OH was prepared according to Deber¹⁸ and packed into cartridges in 2.0-mmol amounts after the purity was checked in the usual manner. Boc-Tyr(2-BrZ)-PAM-resin (0.877 g, 0.57 mmol/g substitution) obtained from ABI was used for the starting material. Other reagents and solvents used for peptide synthesis were obtained from ABI. 1-Hydroxybenzotriazole (HOBt) and *N,N'*-dicyclohexylcarbodiimide (DCC) were each supplied as a 0.5 M solution in *N,N'*-dimethylformamide (DMF) and dichloromethane (DCM), respectively. For the cleavage reaction, HF (Daikin Kogyo Co.), *p*-cresol, dimethyl sulfide (Tokyo Kasei Kogyo Co.), *p*-thiocresol, ether (Nakarai Chemicals Co.), methyl ethyl sulfide, and anisole (Fluka) were used as received. The reagents and solvents used for downstream processing of the crude peptide were analytical grade and purchased from E. Merck and Fluka. Water was prepared through a Milli-Q apparatus (Millipore). Acetonitrile (J. T. Baker) was HPLC grade. Peptide-chain assembly was performed on a 0.5-mmol scale on an ABI Model 430A synthesizer. The synthetic operations were microprocessor controlled and were driven by ABI's VERSION 1.20 software disk. In the synthesis of pCDD(99–126), the quantitative ninhydrin reaction was routinely used to monitor the extent of coupling after each cycle. According to this method, additional couplings were performed as described in the main text to yield 1.95 and 2.00 g of the protected peptidyl resin in two separate runs, respectively. In the synthesis of pCDD(83–126) and pCDD 88, the assembly was based on the version 1.20 double-couple cycle with a few modifications. Following the double coupling, 25% acetic anhydride in DCM was added three times (20, 20, and 30 s, respectively). The capping reaction proceeded for 10 min, and the resin was washed four times (93, 96, 88, and 72 s) with DCM. Sampling was deleted after incorporation of position 72 (Leu) to avoid the loss of peptidyl resin. HPLC instrumentation consisted of Constamatic pumps (I and IIG), a Milton Roy chromatographic control module, an ERC-7211 spectrophotometer (Erma Inc.), and a PM8252 dual pen recorder (Phillips). HPLC conditions were described in the main text. SDS-PAGE was carried out according to the method of Swank and Munkres.¹³ A PMW electrophoresis calibration kit (Pharmacia) and a SE-400 vertical slab unit (Hofer Scientific Instruments) with Consort E425 power supply (Orpegen) were used. Reagents were obtained from Fluka. Sequencing was performed with an ABI Model 470A in combination with an online ABI Model 120A PTH analyzer according to the manufacturer's protocols. Amino acid ratios after acid hydrolysis were determined by use of a Pico-Tag system (Waters).

Synthesis and Purification. pCDD(99–126)/ α -hANP. The *N*^α-Boc group of the protected peptidyl resin corresponding to pCDD(99–126) (1.0 g) was removed by using the "end-nh2" cycle on the synthesizer. The deprotected resin was allowed to stand at room temperature with anisole (1 mL) and methyl ethyl sulfide (0.5 mL) under N₂ for 30 min in an HF reactor (Peptide Institute Inc.) and then stirred with HF (20 mL) at 0 °C for 60 min. After removal of HF *in vacuo*, the residue was

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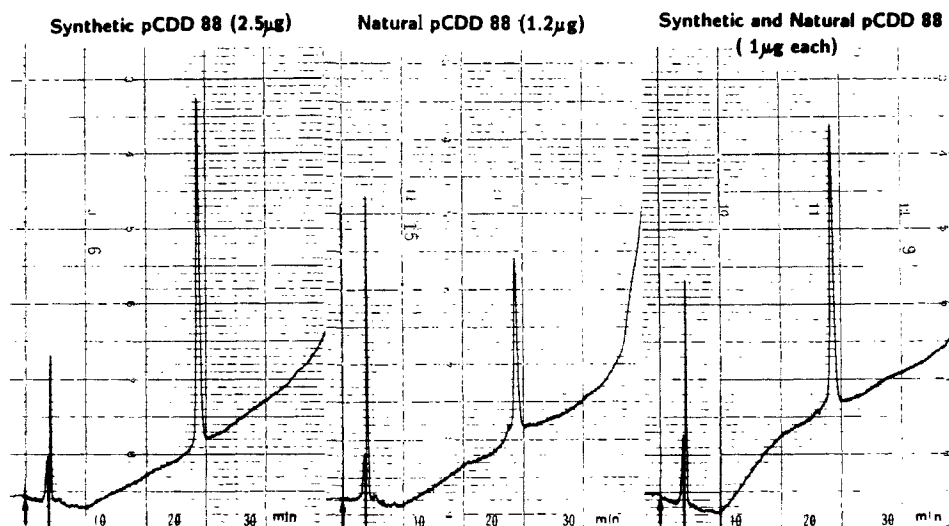


Figure 7. HPLC of synthetic and natural pCDD 88 (same HPLC condition as in Figure 6).

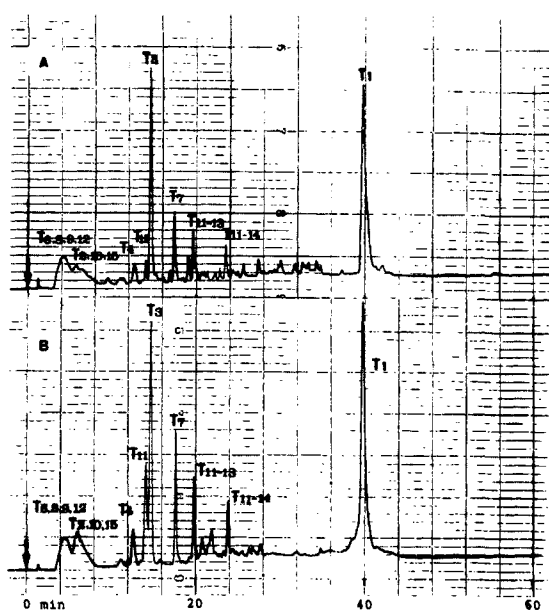


Figure 8. HPLC profiles of tryptic digest. A: 38 μg of natural pCDD 88. B: 47 μg of synthetic pCDD 88. Column, TSK-GEL ODS 120T 4.6 \times 250 mm; eluent, 0.01 M HCl/CH₃CN, 90/10–50/50 (v/v) in 60 min; flow rate, 1.0 mL/min; absorbance, 210 nm.

washed with dried ethyl acetate (150 mL). The peptide was extracted with degassed cold 2 N AcOH (50 mL). The solution was passed through Dowex 1 \times 2 (AcO⁻) (Serva; 10 mL) and freeze-dried. The lyophilizate was dissolved in degassed 1 M NH₄OAc/8 M urea (200 mL) and adjusted to pH 7.4 with 2 M NH₄OH. With stirring, 0.1 M K₃-Fe(CN)₆ in 1 M NH₄OAc (pH 7.4; 8 mL) was added dropwise. The mixture was stirred for a further 40 min and then adjusted to pH 5.0 with AcOH. Half of the resulting solution was desalted on Sephadex G25 fine (Pharmacia) (3 \times 105 cm) with 1 M AcOH as the eluent. The major

peak was freeze-dried and rechromatographed on Sephadex G50 fine (Pharmacia) (2.1 \times 116 cm) by use of 1 M AcOH. The major fractions (3 kDa on SDS-PAGE) were pooled and lyophilized (168 mg). Another half of the oxidized material was filtered through an Amicon YC05 membrane using a Model 8200 cell (Amicon), to remove most of the urea and salts, and then chromatographed as above (128 mg). The resulting material was finally purified on reverse-phase HPLC (yield 20–30%).

pCDD(83–126). After assembly up to position 83, the synthesis was interrupted. The yield of the protected peptidyl resin was 1.73 g, which was derived from 1.95 g of the peptidyl resin corresponding to pCDD-(99–126). A part of the resulting resin (0.50 g) was treated with HF (10 mL) containing anisole (0.5 mL) and methyl ethyl sulfide (0.25 mL) and washed with ether. The peptide was extracted with 2 M AcOH and freeze-dried as described before to provide crude material (153 mg). The resulting material (50 mg) was dissolved in degassed buffer (3.0 mL) consisting of 6 M guanidine hydrochloride, 0.5 M tris(hydroxymethyl)aminomethane (Tris), and 2 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.1). Dithiothreitol (70 mg) was added to the solution under N₂ flashing and the mixture allowed to stand at room temperature for 22 h under N₂. The resulting mixture was desalted on Sephadex G25 fine (3 \times 105 cm) in 1 M AcOH. The major fractions were pooled (ca. 100 mL) and diluted with 330 mL of degassed water, the pH was adjusted to pH 7.6 with 2 M NH₄OH, and the resultant mixture was gently stirred at room temperature for 3 days in an open vessel. Termination of disulfide bond formation was determined according to Ellman.¹² The oxidized mixture was freeze-dried and chromatographed on Sephadex G25 fine (3 \times 105 cm) with 1 M AcOH as the elution solvent. The major fractions were pooled and lyophilized (27 mg). The lyophilizate was further purified on reverse-phase HPLC (yield ca. 15%).

pCDD 88. Starting from the peptidyl resin corresponding to pCDD-(83–126) (1.14 g), additional elongation afforded the peptide-bound resin corresponding to pCDD 88 (1.10 g). An aliquot of this material (363 mg) was N^α-deprotected and transferred to the HF reaction vessel. To this were added *p*-thiocresol (0.4 mL), *p*-cresol (1.6 mL), and dimethyl sulfide (13 mL), and the resultant mixture was allowed to stand at room temperature for 30 min under N₂. After HF (5 mL) was introduced at -70 °C, the mixture was stirred at 0 °C for 2 h (cleavage in low concentration of HF). Following removal of the HF and dimethyl sulfide in high vacuum, the residue was washed with ether (150 mL) and dried

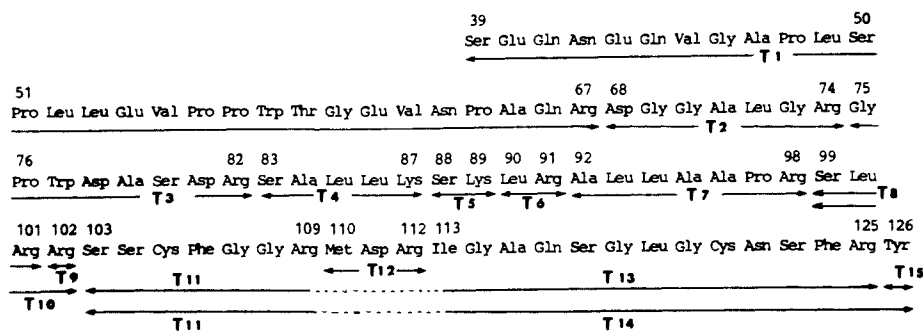


Figure 9. Tryptic fragments of pCDD 88.

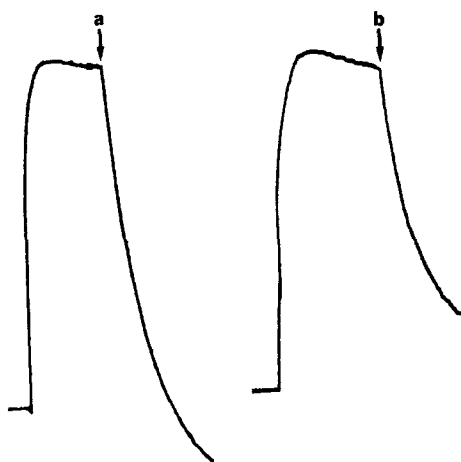


Figure 10. Rabbit aorta relaxant activity of pCDD 88. Left: synthetic pCDD 88. Right: natural pCDD 88.

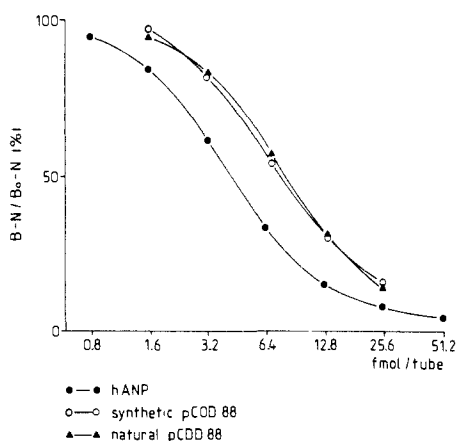


Figure 11. Dose-response curves of synthetic and natural pCDD 88 in α -hANP RIA.

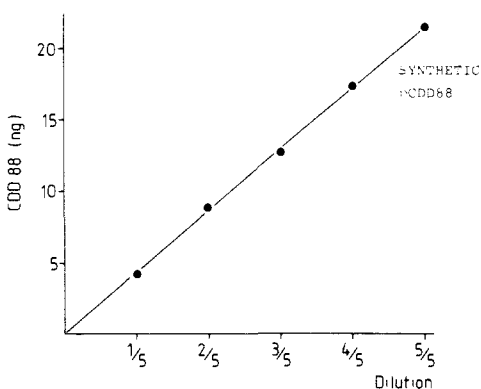


Figure 12. Serial dilution curve of synthetic pCDD 88.

over KOH and P_2O_5 in vacuo for 1 h. The mixture was combined with *p*-cresol (1 mL) and treated with HF (9 mL) for 1 h at 0 °C (cleavage in high concentration of HF). After HF was removed in vacuo, the residue was washed with ether (150 mL), and the peptide was extracted with degassed chilled 2 M AcOH (50 mL) followed by water (50 mL). The combined aqueous extracts were diluted with degassed 6 M guanidine hydrochloride (250 mL) and water (250 mL) and the pH adjusted to pH 7.6 with 28% NH_4OH solution. The resultant solution was further diluted with water to a total volume of 750 mL and was gently stirred in an open vessel at room temperature. Occasional SH determination was performed, and after 3 days, 2% of the starting thiol remained. The solution was dialyzed with Spectra/Por 6 against chilled 0.1 M AcOH (10 L \times 3) and then water (10 L \times 3) at 4 °C. After lyophilization of the dialysate, the residue was chromatographed on Sephadex G50 fine (2.1 \times 116 cm) with 1 M AcOH as an elution solvent. The major fractions (9 kDa as a major component on SDS-PAGE) were pooled (fractions 35–45) and lyophilized to provide the partially purified pCDD 88 (36 mg). This material was further purified in two steps on re-

verse-phase HPLC (yield ca. 10% based on the Sephadex G50 material). The partially purified pCDD 88 (15 mg) was further purified on CM 52 (10 \times 52 cm) by gradient elution from 5 mM and 0.5 M NH_4OAc (pH 4.5) at a flow rate of 15.6 mL/h for 24 h. Each fraction (5.2 mL) was monitored by its absorbance at 280 nm (ELISA as well as HPLC). The desired material eluted at \sim 150 mM NH_4OAc concentration. However, the HPLC profiles did not show an effective purification. The above partially purified pCDD 88 (2 mg) was applied to a Mono-P HR5/20 column (Pharmacia). With 0.075 M Tris acetate (pH 9.3) as starting buffer, the material was eluted with Polybuffer 96 (Pharmacia) at a flow rate of 0.7 mL/min for 100 min. Each fraction (5.0 mL) was tested as above, and the desired peptide-containing fractions were pooled and finally purified on reverse-phase HPLC (yield ca. 100 μ g). Purification on an immunoaffinity column prepared from monoclonal antibody against natural pCDD 126 (NA2F12)⁸ coupled to Affi-gel 10 (Bio-Rad) was also attempted. The details of the preparation of immunoabsorbents and their applications will be published elsewhere. The partially purified pCDD 88 (1.5 mg) was dissolved in PBS buffer and subjected to the above immunoabsorbent (1 \times 10 cm). The desired peptide was eluted with 1 M AcOH. The eluate was further purified on Sephadex G75 (Pharmacia; 1.3 \times 95 cm) followed by reverse-phase HPLC (yield 100 μ g).

Trypsin Digestion. TPCK-treated trypsin (Worthington) in 1% NH_4HCO_3 (4–5 μ g in 2 μ L) was added to the peptide solution (1 μ g/ μ L) and incubated at room temperature for 4 h and at 95 °C for 5 min. After lyophilization, the tryptic digests were separated on reverse-phase HPLC. Each peak was analyzed by sequencing and amino acid analysis.

Bioassay. After an equilibration period of 2 h in a standard physiological saline, helical strips (ca. 2 mm \times 10 mm) of rabbit (New Zealand White) aorta were precontracted by 10^{-4} M noradrenaline. After the contraction had been stabilized, peptide samples dissolved in the same saline were added. Vasorelaxation was recorded by amplification.

Immunoassays. Radioimmunoassay (RIA) was carried out according to the following protocol: Sample (50 μ L) was incubated with anti-serum MCR314 (dilution 1:8000; 50 μ L) in assay buffer consisting of 50 mM sodium phosphate buffer containing 0.5% bovine serum albumin (BSA), 10 mM EDTA, and 0.1% Triton X-100 (pH 7.4) at 4 °C for 24 h. Tracer, 3-[¹²⁵I]iodotyrosyl²⁸- α -hANP (IM187, Amersham; 50 μ L), was then added to the assay buffer and the mixture further incubated at 4 °C for 24 h. After incubation with normal rabbit serum (dilution 1:100; 50 μ L) and a second antibody (Cappel) (dilution 1:80; 50 μ L) at 37 °C for 3 h, the mixture was centrifuged (3000 rpm) at 4 °C for 30 min. After aspiration of the supernatant, radioactivity was counted with a γ counter (Packard, Autogamma 5110).

The two-site ELISA was carried out as follows: A purified antiserum NAHD-16 (0.1 mL, 10 μ g/mL), which was obtained from antiserum generated against synthetic α -hANP by using protein A-Sepharose CL-4B (Pharmacia) in the conventional manner, was adsorbed onto 96-well microtiter plates (Immuno Plate II F, Nunc) at 4 °C for 24 h in 10 mM phosphate saline buffer (PBS). After being washed three times with a washing buffer of PBS containing Tween 20 (PBS-T), standards or samples diluted in PBS containing 0.5% BSA (PBS-A; 0.1 mL) were added to the above plates. After incubation at 37 °C for 2 h followed by washing with PBS-T as above, this was then incubated with NA2F12 (10 μ g of IgG/mL) in PBS (0.1 mL) at 37 °C for 2 h. After a wash with PBS-T as above, anti mouse Ig-horseradish peroxidase conjugate (P161, Dakopatts) (0.1 mL; dilution 1:10000) in PBS-A was added. After further incubation at 37 °C for 1 h followed by a wash with PBS-T, a substrate solution (0.1 mL) consisting of *o*-phenylenediamine hydrochloride (Fluka; 3 mg/mL), 0.01% H_2O_2 in 0.1 M citric acid, and 0.2 M Na_2HPO_4 (pH 5.0) was added. After 30 min at room temperature, the enzyme reaction was stopped with 2 M H_2SO_4 (0.1 mL). The absorbance was measured at 492 nm with a Titertek Multiscan (Flow Laboratories).

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Registry No. pCDD(99-126) (oxidized), 89213-87-6; pCDD(83-126) (oxidized), 112509-58-7; pCDD(88) (oxidized), 116887-28-6; pCDD(99-126) (reduced), 91917-63-4; pCDD(83-126) (reduced), 116887-25-3; pCDD(88) (reduced), 116887-29-7; BOC-Asp(OcHx)-OH, 73821-95-1; BOC-Pro-OH, 15401-08-8; BOC-Arg-OH, 13726-76-6; BOC-Phe-OH, 13734-34-4; BOC-Ser-OH, 3262-72-4; BOC-Asn-OH, 7536-55-2; BOC-Cys-OH, 20887-95-0; BOC-Gly-OH, 4530-20-5; BOC-Leu-OH, 13139-15-6; BOC-Gln-OH, 13726-85-7; BOC-Ala-OH, 15761-38-3; BOC-Ile-OH, 13139-16-7; BOC-Asp-OH, 13726-67-5; BOC-Met-OH, 2488-15-5; BOC-Lys-OH, 13734-28-6; BOC-Trp-OH, 13139-14-5; BOC-Val-OH, 13734-41-3; BOC-Glu-OH, 2419-94-5; BOC-Thr-OH,

2592-18-9; BOC-Pro-OH, 15761-39-4.

Supplementary Material Available: Figures 13-18 showing elution profiles of synthetic pCDD(99-126) on Sephadex G50 fine and in HPLC, the rabbit aorta relaxant activity of purified synthetic pCDD(99-126), the gel filtration profile of synthetic pCDD 88 on Sephadex G50 fine, the elution profiles of synthetic pCDD 88 after immunoaffinity purification in HPLC, and a standard curve for pCDD 88 in two-site ELISA (4 pages). Ordering information is given on any current masthead page.

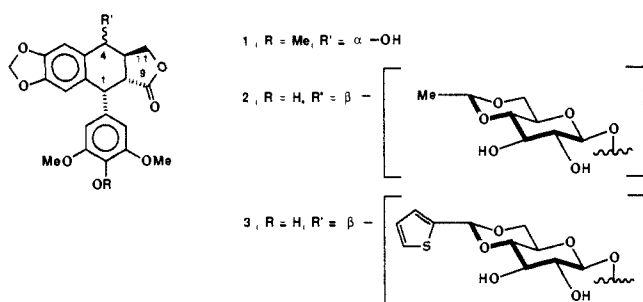
Asymmetric Total Synthesis of (-)-Podophyllotoxin†

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Contribution from the Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523. Received May 2, 1988. Revised Manuscript Received June 27, 1988

Abstract: Using a diastereoselective addition of the appropriate aryllithium to a naphthalene-containing chiral oxazoline leads to advanced intermediate **11** in the podophyllotoxin series. The latter is obtained in a 92:8 de. Transformation of the oxazoline moiety to the requisite lactone **18** followed by invoking the Kende route to the target gave natural (-)-podophyllotoxin in 94% ee. The overall yield of the sequence, accomplished in 24 steps, was 5%.

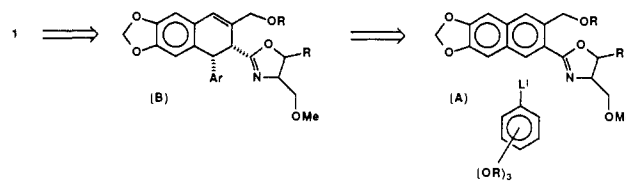
The aryltetralin lactone podophyllotoxin (**1**) occupies a unique and significant place among lignan natural products since its recognition as a potent antitumor agent when affixed to a glucopyranose moiety.¹ Thus, Etoposide (**2**) and Teniposide (**3**) are



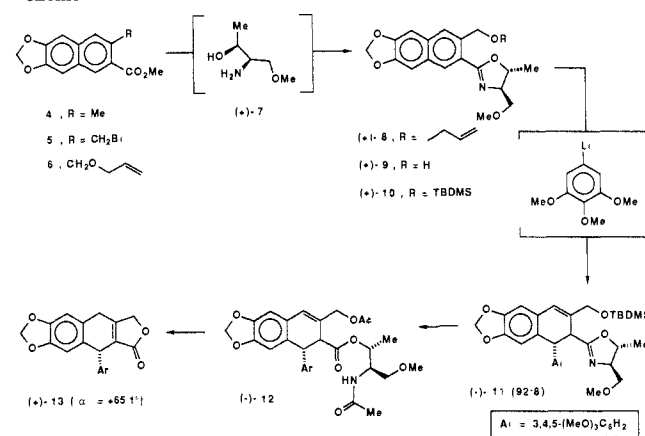
currently in the armory of antitumor drugs.² Although the natural podophyllin resin was used in folk medicine,³ it was not until the 1940s that its antitumor activity was confirmed and this triggered intense studies toward synthetic routes led mainly by the late Professor Walter Gensler.⁴ Gensler's contributions⁴ in the 1950s and 1960s on synthetic, structural, and mechanistic aspects of podophyllotoxin provided much of the basis for the synthetic studies that followed.⁵ All the reported synthetic routes to **1** produced racemic material or involved classical resolution techniques.⁶ Our current studies on the asymmetric tandem addition to chiral naphthalenes⁷ (Scheme I) appeared to provide a very attractive route to chiral lignans and, in particular, podophyllotoxin. Thus, diastereoselective addition of an aryllithium to an oxazoline-containing naphthalene (**A**) would, in principle, provide an adduct (**B**) that could be elaborated with the proper substituents. Removal of the chiral oxazoline in **B** would lead to (-)-podophyllotoxin (**1**).

The synthetic route originated (Scheme II) with the naphthoic ester **4**, prepared earlier in our laboratory.⁸ Bromination using

Scheme I



Scheme II



NBS-AIBN (CCl_4 , 70-74 °C) proceeded to give **5** (86%). The allyl ether **6** was prepared (92%, NaH, allyl alcohol) and then

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† This paper is dedicated to the memory of Professor Walter J. Gensler (1917-1987).