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DESIGN AND SYNTHESIS OF DNA SEQUENCE SELECTIVE HEAD-TO-TAIL LINKED BIS-LEXITROPSINS

BY

DEQI GUO

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF CHEMISTRY

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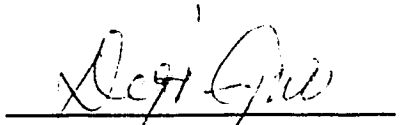

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled DESIGN AND SYNTHESIS OF HEAD-TO-TAIL LINKED BIS-LEXITROPSINS submitted by DEQI GUO in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE IN CHEMISTRY.

M. Palcic

E. Knaus

J. W. Lown (Supervisor)

Date : April 29, 1993

Abstract

A group of head-to-tail linked bis-lexitropsins designed to examine phasing aspects of DNA binding has been synthesized in which the linker consists of polymethylene groups $-(CH_2)_n-$ with n = 1 to 7. Bindings of these agents to poly(dA-dT) are either stronger than (n = 2, 4, 6) or comparable (n = 1, 3, 5, 7) with distamycin. Complementary strand footprinting provides evidence of effective bidentate binding within suitable AT-rich tracts covering 10 ± 1 bp for n = 2, 4, and 6 but not for n = 1, 3, 5, 7 which only engage in monodentate binding wherein one lexitropsin moiety at a time is bound. Cytostatic activity against KB cancer cell and *in vitro* activity against a range of viruses are reported. Excellent correlation is observed between effective binding and anticancer cytostatic potency for this homologous series. The fact that the even-numbered linked agents are more potent than the odd-numbered homologous indicates effective bidentate DNA binding is a more important contributor to potency than increased lipophilicity. Two head-to-tail rigid-linked bis-lexitropsins have been prepared for a preliminary biological evaluation.

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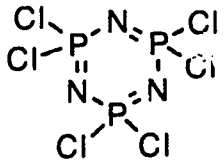
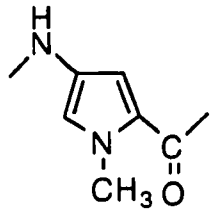
Abbreviations

$A_x a$	straight chain ω-amino acid with x carbon atoms
b	broad
bp	base pair
CDI	1,1'-Carbonyldiimidazole
d	doublet
DCC	1,3 - Dicyclohexylcarbodiimide
dec.	decomposed
DMAP	4-Dimethylaminopyridine
EDCI	Ethyl (dimethylaminopropyl) carbodiimide
	CH_3CH_2-N=C=N-$(CH_2)_3$-N$(CH_3)_2$•HCl
FAB-MS	Fast atom bombardment mass spectrum
m	multiplet
$(NPCl_2)_3$	Phosphonitrilic dichloride cyclic trimer



PAH	propionamidine hydrochloride
PN	propionitrile

Pyr (or Py)



q	quartet
s	singlet
t	triplet

Chapter 1
Introduction

I Alternative Approaches to the Development of Sequence Specific DNA Effectors

Even today, new drugs are usually not discovered by rational design. On average, about 10000 new compounds have to be synthesized and tested in order to discover a new active substance worth development. In many cases the active substance is directed against proteins such as enzymes, receptors or ion channels, the structure and mode of action of which are usually very complicated and often incompletely understood (Fig. 1 a). Another problem associated with the use of many drugs in the treatment of human diseases is of specificity. Many antineoplastic and antiviral agents such as ribavarin, acyclovir, vidaravine, zidovudine, idouridine, trifluridine, and amantidine interfere with replication and transcription of DNA.[1] Antisense oligonucleotides seem promising in selective intervention at the level of the nucleic acid (Fig.1 b),[2,3] in practice it is plagued with difficulties associated with cellular uptake, their intracellular instability and subcellular distribution, and diastereomer problems associated with backbone modification.[4] An additional problem has been the resistance of antisense α-DNA:mRNA hybrids to RNase H degradation.[5] Nevertheless, the antisense strategy for chemotherapeutic agent development is being pursued vigorously on an international scale. So far interest has been confined to antivirals wherein viral RNA has been the predominant target. For virus that has integrated into host DNA , such as HIV-I and herpes, this approach may only serve to control the spread of the virus but is unlikely to solve the underlying problem of latency. Prolonged treatment would be required with the attendant problems of side effects and development of resistance to the drug.[6]

A complementary approach in genetic targeting to the antisense oligonucleotide is that of antigene agents which is designed to target individual cellular or proviral gene sequences

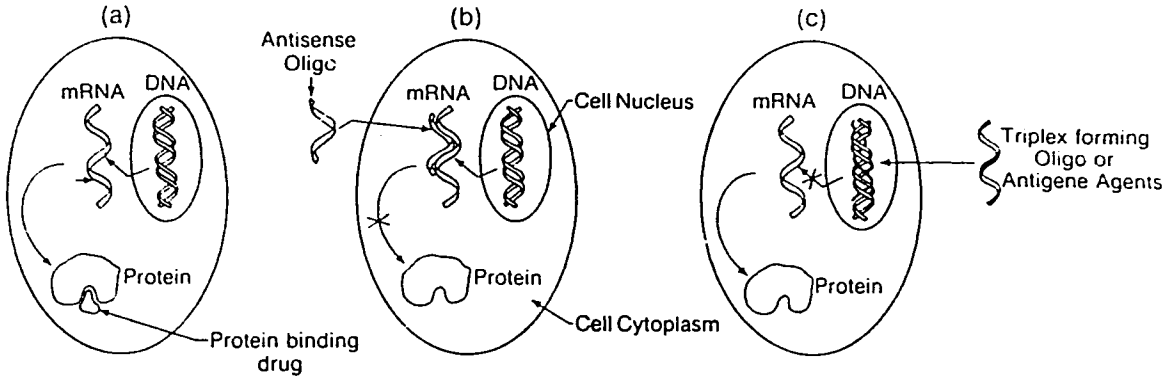


Figure 1 Schematic representation of alternative strategies of chemical intervention in the cell cycle.(a) Introduction of protein-binding drug, (b) antisense oligomer approach, (c) triplex formation or introduction of a groove binding antigene agent.

(Fig. 1 c).[7-13] On transcription, every gene gives rise to a relatively large number copies of mRNA, which is then translated to a large number of protein molecules. Thus, if the encoding gene is transcriptionally active, RNA species typically exist at a copy number of 100-100,000 per cell.[14] However, at the DNA level, the copy number of the corresponding sequence is only a few per cell, moreover, DNA is only being regenerated at the modest rate of cell division. Thus DNA directed agents could potentially be effective at lower dose levels than those of the corresponding antisense or conventional antimetabolite agents.

Current efforts within the framework of antigene strategy are concentrated on triple-helix motif for sequence-specific DNA recognition. Pyrimidine oligodeoxyribonucleotides bind purine tracts in the major groove of DNA parallel to the purine Watson-Crick strand through the formation of specific Hoogsteen hydrogen bonds to the purine Watson-Crick base.[12] Specificity is derived from thymine (T) recognition of adenine•thymine (AT) base pairs (TAT triplet) and N_3-protonated cytosine (C^+) recognition of guanine•cytosine (GC) base pairs (C^+GC triplet) (Fig.2). The purine and the pyrimidine motifs both require homopurine tracts with DNA, thus greatly limiting the number of targets that can be addressed. Circumvention of this code-restriction will require devising novel heterocycles that can engage in non-natural base triplet formation.[15] Other problems associated with antigene strategy are low chemical yield (15-25%) for double-strand oxidative cleavage by oligonucleotide equipped with EDTA•Fe(II)[16] and limited number of available enzymatic cleavage sites.[11]

II Prototype Minor Groove Alkylators and Their Development

An alternative and complementary approach to the antisense and antigene efforts is to develop sequence specific probes based on naturally occurring oligopeptides netropsin 1 and distamycin 2 (Fig. 3).[17-19] Deoxyribonucleic acid consists of AT and GC base pair held together through hydrogen bond like rungs of twisted ladder, and the base sequence

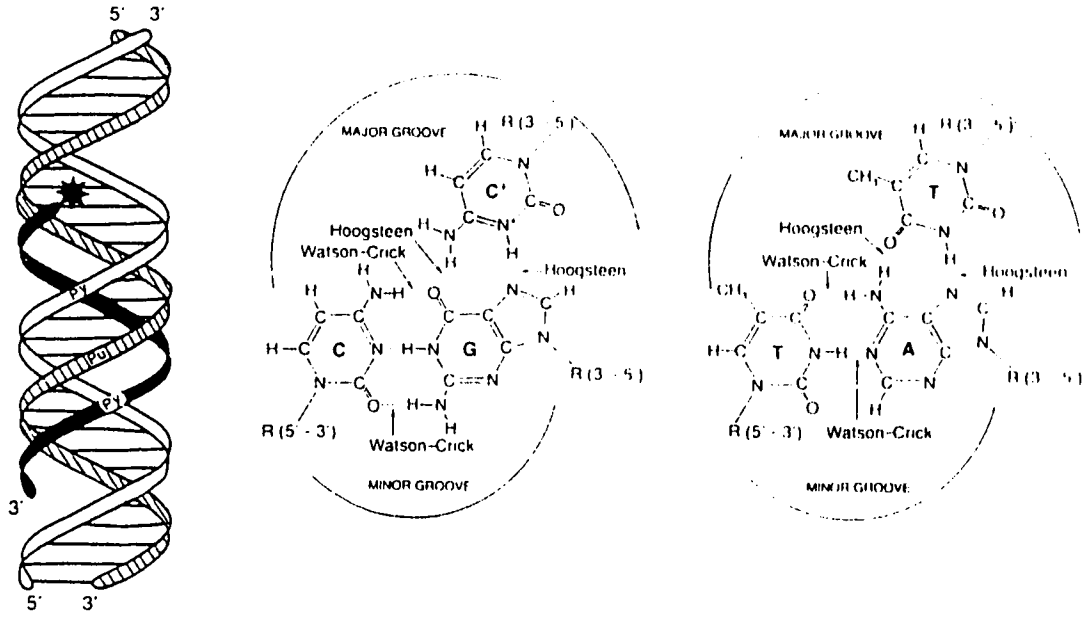


Figure 2 . Oligonucleotide binding to duplex DNA. Left : the third strand homopyrimidine oligonucleotide (black) binds to the major groove of duplex DNA at a homopurine.homopyrimidine sequence. The star represents a reactive group that can induce irreversible reactions on both strands of DNA. Right : Hoogsteen hydrogen bonding of T and protonated C to A.T and G.C Watson-Crick base pairs, respectively.

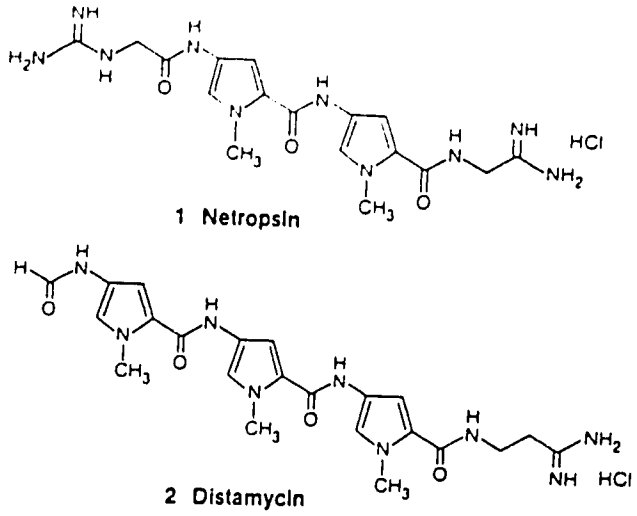


Figure 3 Structures of natural antibiotics netropsin(1) and distamycin(2).

information of B-DNA can be stored in the local structure of double helix.[20] DNA contains two channels of information, the major groove and the minor groove (Fig.2). The major groove is richer in information, as defined by the number and discriminatory capacity of hydrogen bond acceptor and donor sites, compared with the minor groove. In addition, the depth and width of the major groove are about 1 and 3.5 Å respectively which are greater than those of the minor groove. Therefore, bulky repressors or control proteins generally bind in the major groove.[21] On the other hand, the minor groove, which represents a vulnerable site of attack, is generally preferred by many low molecular weight antiviral, antibiotic, and anti-cancer agents. In terms of the design of sequence-reading molecules, while intercalators show little base preference, groove-binding agents of natural origin such as netropsin and distamycin often display strict sequence-recognizing properties. X-ray[22] and NMR[23] studies of netropsin-DNA and distamycin-DNA complexes reveal that the crescent-shaped di- and tripeptides are bound in the middle of the minor groove of an AT rich sequence. The amide hydrogen of the N-methylpyrrolecarboxamides form bifurcated

hydrogen bonds with N_3 of adenine and O_2 of thymine on the floor of the minor groove (Fig.4 a). Footprinting studies by Dervan and co-workers shows that distamycin binds to $(AT)_4$ in the minor groove of the DNA and consequently leads to a general rule of a ligand bearing n amide linkers affording a binding site size of n+1 base pairs.[24] Rational structural modification of netropsin and distamycin led to the development of lexitropsins, or information-reading agents,[18,19] some of which are capable of recognizing unique sequence and they show no memory for the preferred sequence of the parent antibiotics.[25]

Recent evidence suggests that under certain conditions distamycin forms a 2:1 complex containing two ligand molecules per duplex in which the two ligands align side by side in an antiparallel fashion.[26] The amidinium side chains of the two ligands in such complexes extend into GC base pair around the central AT rich region where the minor groove is wider and thus accommodates the bulky portions of the molecules. More recent studies reveal that a certain lexitropsin, obtained by replacing the central N-methylpyrrole unit by an N-methylimidazole ring , also forms a 2:1 complex incorporating the GC base pair in the middle of an AT tract, thereby increasing the width of the minor groove in the central section of the oligomer sequence.[27] Dervan and coworkers have also recently reported two 2:1 complexes where pyridine-2-carboxamide-netropsin (2-PyN) and 1-methylimidazole-2-carboxamide-netropsin (2-ImN) bind specifically to the five base pair sequence 5'-TGTCA-3' by a side-by-side antiparallel motif (Fig.5).[28]

III Lexitropsins - information-reading Oligopeptides and Their Design

Analysis of the X-ray data of netropsin and distamycin binding to DNA suggests that the main contributors to molecular recognition consist of (1) hydrogen bonds from the amide NHs to the floor of the minor groove, (2) electrostatic interactions between the cationic termini and the negatively charged regions of the minor groove, (3) van der Waals contacts between the methylene of the propionamidine side chain, ring CH's on the ligand

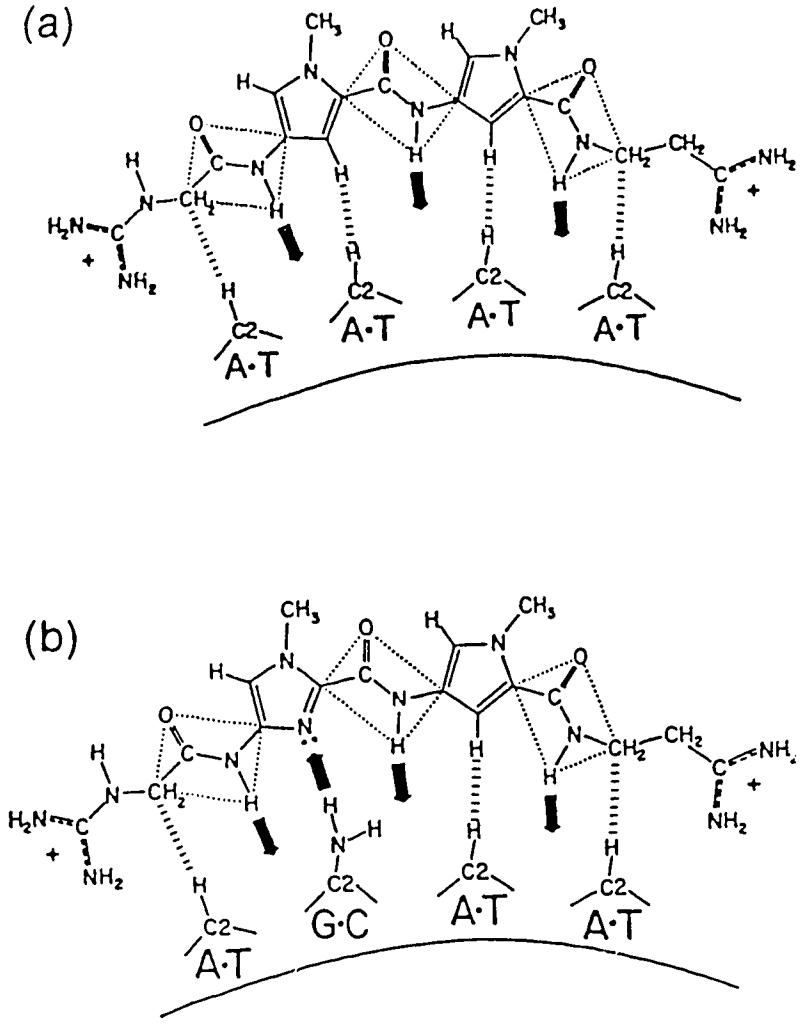


Figure 4 (a) Bonding components contributing to the molecular recognition and sequence selective binding of netropsin to $(AT)_4$, and (b) Prediction of rational alternation of DNA sequence recognition by a prototype lexitropsin.

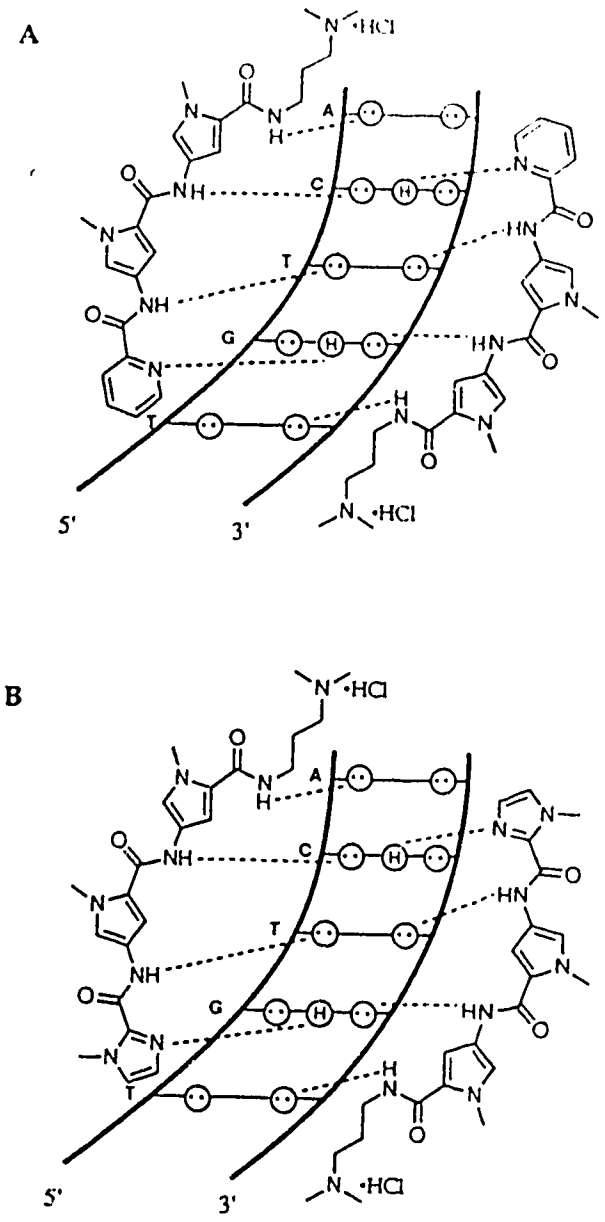


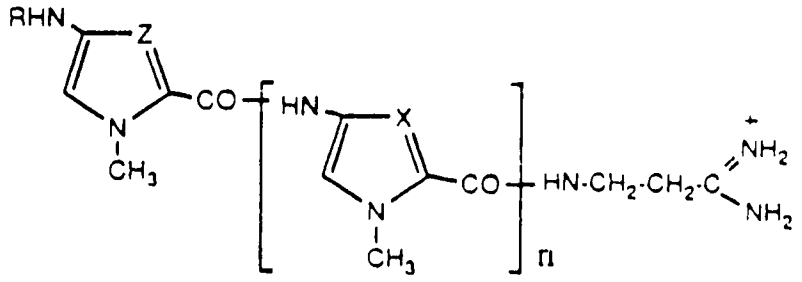
Figure 5 2:1 Binding models for the complexes formed between (a)2-PyN and (b) 2-ImN with the 5'-TGTCA-3' sequence. Circles with dots represent lone pairs on N_3 of purines and O_2 of pyrimidines. Circles containing H represent the N_2 of guanine. Putative hydrogen bonds are illustrated by dashed line.

and purine, pymidine moieties on DNA. From the base of this analysis evolves the concept of lexitropsin wherein the netropsin structure was modified to accommodate an appropriate heterocyclic moiety (e.g. imidazole, thiazole, triazole) so that the lone pair electrons of the heteroatoms positioned in the minor groove can hydrogen-bond to the 2-NH_2 group of guanine, thus permitting a change in the base site from AT to GC (Fig.4 b).

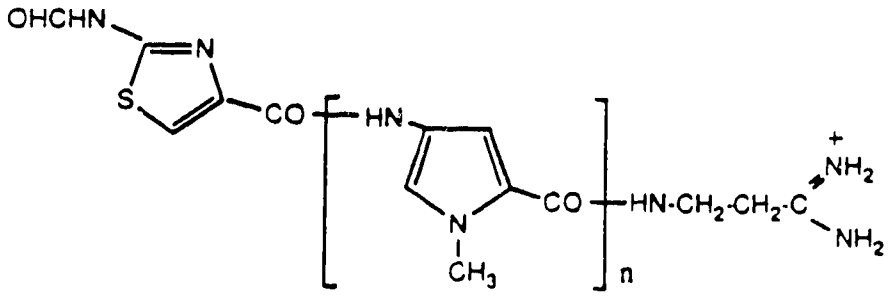
From statistical considerations of base content and genomic sizes, it can be estimated that a unique sequence in the human genome is 15-16 base pairs.[30] One goal therefore is to be able to target ligands selectively to a sequence of this size. Experiments with spin labelled lexitropsins in KB human cancer cells reveal rapid uptake, reasonable intracellular stability and concentration of drugs in the nucleus. Systematic exploration of the characteristics of different types of lexitropsins indicates a number of structural and stereochemical features that may contribute to drug design as follows.

III A Molecular Electrostatic

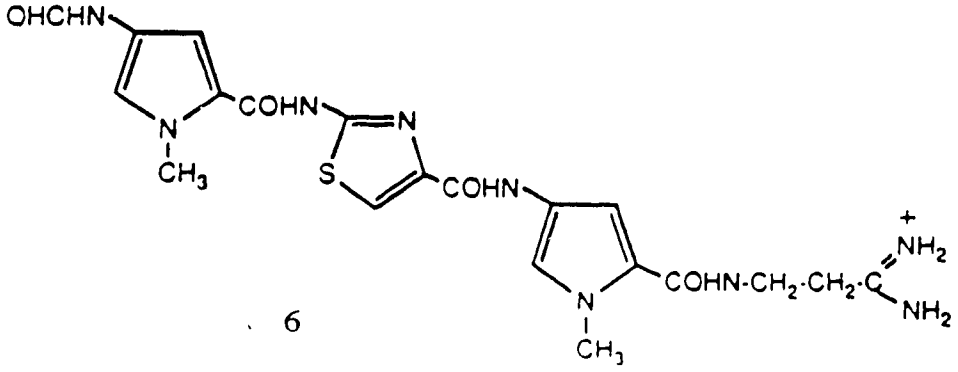
The first generation lexitropsins with one or more imidazole rings in place of N-methylpyrrole units in netropsin were synthesized.[32] Although these biscationic lexitropsins show a greater tolerance of GC bases than netropsins, they retain overall AT base selectivity. Theoretical studies reveal that the AT region in the minor groove has a deeper negative potential than GC rich regions.[33] The calculations by Zakrewska[34] and Pullman[35] suggest that a positively charged ligand would be attracted to the negatively charged region at the bottom of the minor groove rather than to the phosphate backbone. As a result, both biscationic netropsin and related prototype lexitropsins preferentially bind to AT rich regions in the minor groove. Therefore, a second generation of lexitropsins with N-formyl group in place of guanidino acetic acid side chain so as to reduce the overall cationic charge were synthesized (Figure 6).[36] These lexitropsins therefore resemble distamycin with one positively charged group. DNase I footprinting studies on a restriction



3 Z = X = N, n = 1 R = CHO



4 n = 1; 5 n = 2



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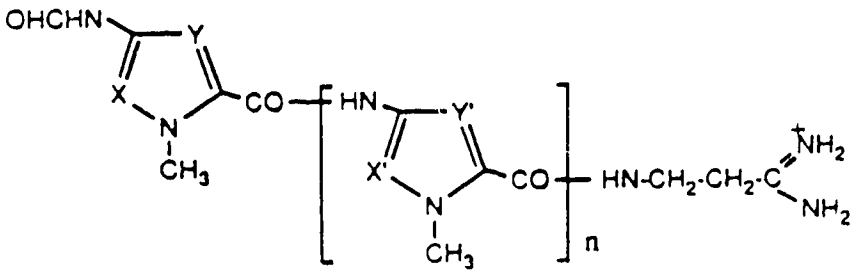
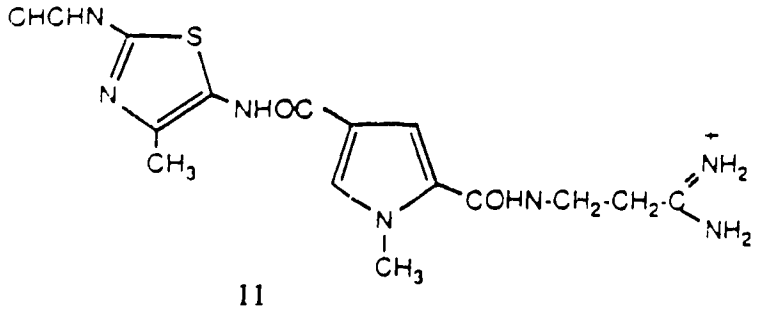


7 X = Y = CH, X' = Y' = N, n = 1; 8 X = Y = N, X' = Y' = CH, n = 1

9 X = Y = X' = Y' = N, n = 1; 10 X = Y = N, X' = Y' = CH, n = 2



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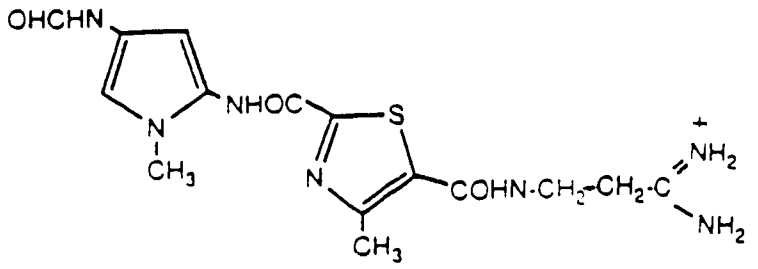

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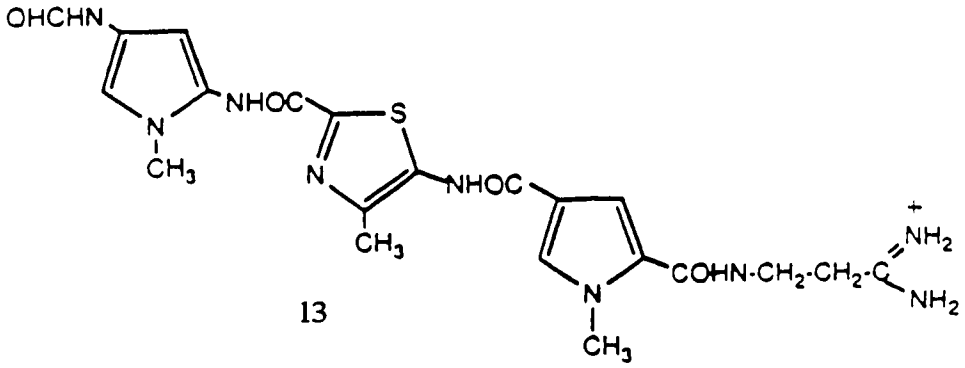


13

Figure 6 Structures of imidazole, thiazole, and triazole containing monocationic lexitropsins (3)-(13).

fragment with these second generation lexitropsins indicated a pronounced change in the sequence specificity from AT base pairs to GC rich sites.[25]

III.B. DNA Base Site Acceptance-Hydrogen Bonding Interactions

Monocationic lexitropsins were therefore shown to exhibit their intrinsic base and sequence preferential binding in contrast to bicationic ligands,[25] and thereby represent better models for the investigation of molecular recognition components. The proton NMR study of binding of bis-imidazole analogue of distamycin 3 (Figure 6) to d(CATGG CCATG)$_2$ shows that the compound is located at 5'-CCAT with the formyl terminus towards the 5'-end of the binding site.[36] Studies of the monocationic thiazole lexitropsins with N directed towards the minor groove 4-6 (Figure 6) support the hypothesis that the heteroatom is involved in hydrogen bonding with 2-NH_2 of guanine.[37] Subsequently a series of compounds with triazole units in place of N-methylpyrrole rings 7-10 were prepared and an increased preference for GC sites by these lexitropsins was observed.[38] The comparative UV and CD studies with bis-pyrrole, bis-imidazole, and bis-triazole lexitropsins unequivocally established that the affinity of these heterocyclic carboxamides for d(A-T) bases is in the order pyrrole carboxamides >> imidazole carboxamides > triazole carboxamides.

III. C. Base Site Avoidance-van der Waals Radius of the Heteroatom Facing the Minor Groove

In order to examine the effect of the larger van der Waals radius of sulfur in binding, thiazole containing lexitropsins with the sulfur aligned towards the minor groove of DNA were synthesized 11-13 (Figure 6).[37] These compounds showed greater preference for AT sequences than the isomeric thiazole lexitropsins with sulfur aligned away from the minor groove of DNA and distamycin in MPE•Fe(II) complementary strand footprinting. The GC base avoidance and strict AT base specificity is due to the fact that the more sterically

demanding sulfur orientated on the concave side of the ligand directed towards the floor of the minor groove clashes with exocyclic 2-NH_2 group of guanine.

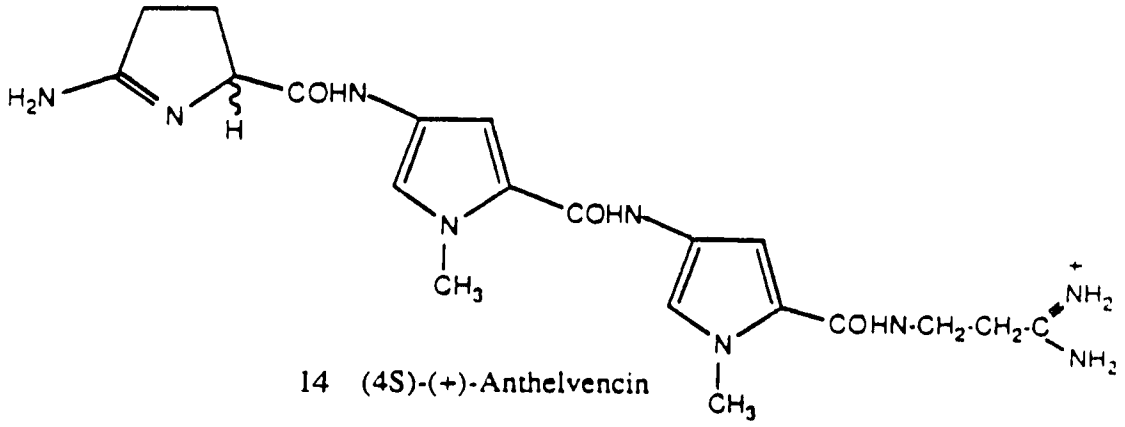
III. D van der Waals Contacts at the 3'-End of the Recognition Site of Lexitropsins

The X-ray analysis of the crystal structure of netropsin and the dodecamer d(CGCGAATTCGCG)$_2$ indicates that methylene of the propionamidine side chain is involved in van der Waals contacts with 2CH of adenine.[22 a,b] NMR studies showed that the interaction between the methylene side chain at the carboxyl terminus of the lexitropsin and DNA is the determining factor in the recognition of 3'-terminal AT base.[39] The fact that the lexitropsin can not bind to a GC site on 3'-end is due to the steric clash between the 2-NH_2 of guanine and the methylene side chain, and the ligand thereby recognizes only 3' AT by default.

III. E Effects of Ligand Chirality on DNA Binding

Since the DNA receptor is chiral, the chirality of ligands may be anticipated to influence binding efficiency. Anthelvencin A exists in two enantiomeric forms: the naturally occurring isomer (4S)-(+)-anthelvencin 14 and unnatural isomer (4R)-(-)-anthelvencin 15 (Fig.7).[40] NOE measurements indicated that the (4S) enantiomer is propeller twisted so that the pyrrole moieties are isohelical to DNA and bind tightly in the minor groove. In contrast, the unnatural enantiomer adopts a coplanar conformation.[41] Molecular modelling studies showed that the (4S)-hydrogen is directed away from the groove and the positively charged 2-amino-1-pyrrolinium group is therefore directed towards the negative potential of AT region for more favorable electrostatic interaction, while the opposite orientation obtain for the (4R)-isomer. In addition, the rate of exchange of the lexitropsins between two equivalent sites via a flip-flop mechanism [18a] as determined by NMR spectroscopy is found to be dependent on the chirality of the oligopeptides.

III. F Phasing between the Ligand and DNA



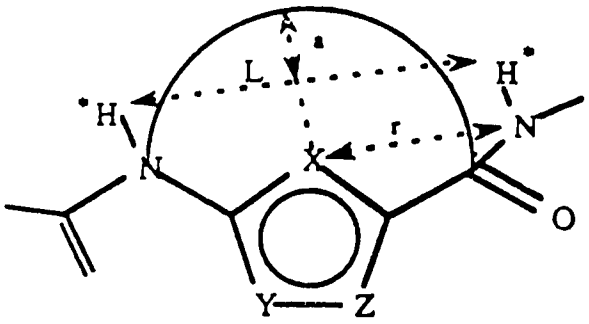
14 (4S)-(+)-Anthelvencin

15 (4R)-(-)-Anthelvencin

Figure 7 Structures of natural (4S)-(+)- and unnatural (4R)-(-)-Anthelvencins(14) and (15).

The distance between the two adjacent amide hydrogens on either side of a heterocyclic unit is designated as the repeat distance l. The repeat distance in different heterocycles has been calculated (Table 1).[42] For an ideal B-DNA with its radius of 4.5 Å (the distance from the helical axis to the amide hydrogen of the lexitropsin) the repeating unit of the perfectly located lexitropsin amide hydrogen, capable of forming H-bonding, can be estimated to be 4.4 Å. As shown in the table, none of the heterocyclic amides have repeat distances to match perfectly with successive DNA bases, and this results in a lack of phasing. The problem of phasing can be alleviated by connecting a heterocyclic unit of smaller l with that of larger l. An alternative approach is to join the heterocyclic amide units with a suitable linker or tether. Molecular mechanics calculations revealed that a number of factors must be considered in selecting an ideal linker, these are : (a) linker length: the tether unit should constrain the binding moieties so that the distance between the amide hydrogens is about 4.4 Å or a multiple of it: (b) flexibility of linker length: an ideal tether should compensate for either too long a ligand unit (e.g. thiazole unit with the sulfur directed towards the floor of the minor groove) or too short a ligand unit (e.g. thiazole unit with the

Table 1 Dependence of Repeat Distance l in Lexitropsins on Heterocyclic Moiety#



r = van der Waals radius of X

	X	Y	Z	r	a	L*
Pyrrole	CH	CH	N	(1.0 ± 0.2)	1.2	4.7
Imidazole	N	CH	N	1.5	0.3	4 2
Thiazole	S	N	CH	1.8	1.5	5.5
Thiazole	N	S	CH	1.5	0.2	4.0
Triazole	N	N	N	1.5	0.3	4.2

#From Prof. J. W. Lown (ref. 42).

*Atoms in common plane.

nitrogen directed away from the minor groove) so as to bring an adjacent lexitropsin back into phase with the base pairs of DNA;[43] (c) correct shape: the tether unit should not interfere with the required crescent shaped conformation of a suitable ligand; (c) rigidity of linker: for too flexible a ligand, a portion of the molecule may remain in the solution or may be involved in a "dancing" mechanism of two site exchange in which the bound and the unbound moieties exchange rapidly to give rise to operational time average bidentate binding that may be as effective as "true" bidentate binding for biological purposes(Fig.8); (e) stability of the required conformation: if the most stable conformation of the tether is the

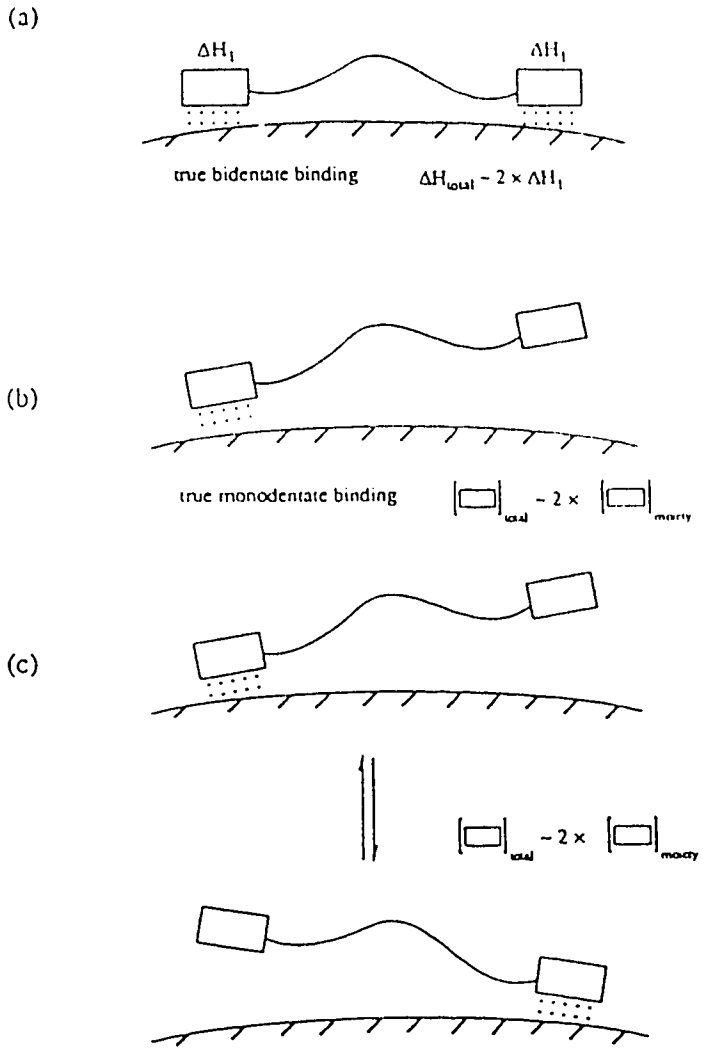


Figure 8 Depiction of alternative modes of binding of potentially bidentate ligands (a) true bidentate binding, (b) true monodentate binding, (c) "dancing" mechanism or operational bidentate binding.

one required for binding, the binding process would be favorable both kinetically and thermodynamically.

Candidate tethers are cis- and trans-1,2-disubstituted cycloalkanes from three to six member rings, Z and E alkenes, and polymethylene linkers. The force field analysis showed that the better tethers among those examined in the studies are trans-cyclopropane, trans-cyclobutane, trans-cyclopentane, the fumaric acid derivatives and the succinate bridge.[42,43]

III. G Amide Isosteres - Thioformyldistamycin

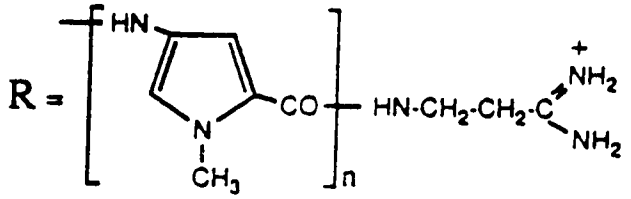
The major problem associated with oligopeptides is their susceptibility to intracellular degradation by peptidases. The terminal formyl amide bond is the most susceptible to acid and peptidase hydrolysis thus is replaced by thioformyl amide bond.[44] Complementary strand MPE-Fe(II) footprinting analysis of distamycin and its thioformyl surrogate on a ECoRI/Hind III restriction fragment of pBR322 DNA revealed that they both occupy the same AT rich sequences of 5 ± 1 base pair long with similar affinity. NMR studies on the free thioformyldistamycin confirm the existence of E and Z conformations with the S atom directed towards (in fresh DMSO) and away from (in aged DMSO) the minor groove respectively. However, no difference in footprinting pattern was discerned. It seems that the Z conformer is forced into the more favorable E conformer in the process of binding to DNA even though the rotational barrier for two conformers is 26 Kcal/mol.[44]

IV Pharmacological Properties of Lexitropsins

IV. A Antiviral and Anticancer Activity

Lexitropsins, like netropsin and distamycin, generally display wide-spectrum antiviral and anticancer activity. Antiviral activity in vitro as well as cytotoxic action against a range of human and murine tumor cell lines for a series of flexible polymethylene linked bis-

netropsin (16-27) (Fig. 9) are listed in Table 2, Table 3 and Table 4.[29,45] Generally the longer the lexitropsin, the more strongly they bind to DNA, the more potent they are in terms of cytotoxicity and inhibitory activity against human tumor cell lines. It was found that for this homologous series a minimum linker length of $(CH_2)_3$ is required for bidentate binding to 11 contiguous base pair.[18b] In order to understand some of the design features that contribute to antiviral potency, the behavior of a series of rigid 1,2-cycloalkane linked bis-netropsins was examined by force field calculations and then tested experimentally by MPE complementary strand footprinting and by antiviral evaluation.[38] The result indicated, as predicted, cis-linked bis-lexitropsins permit only monodentate-DNA binding which is reflected in their relatively lower antivaccinia potency.[46] In contrast, the trans-linked isomers predicted to be more efficient in DNA binding provided evidence of firm bidentate binding(Fig. 8) which is also reflected in a consistently higher antivaccinia potency.[46] Also, as predicted, trans-cyclopropane linked structures are the most potent in their antiviral and anticancer activity.



16 R-CO-R, n = 2;

17 R-COCH$_2$CO-R, n = 2

18 ~ 26 R-CO(CH$_2$)$_{2\text{-}10}$CO-R, n = 2;

27 R-CO(CH$_2$)$_2$CO-R, n = 1

Figure 9 Structures of a series of linked bis-lexitropsins

Table 2. Cytotoxicity and antiviral activity of the linked lexitropsins in primary rabbit kidney(PRK) cell cultures*

cmpd	n[a]	min cytotoxic concn,[b] μg/mL	min inhibitory concn,[c] μg/mL			
			herpes simplex virus 1 (IOS)	herpes simplex virus 2 (G)	vaccinia virus	vesicular stomatitis virus
16	0	≥40	>10	20	7	>40
17	1	≥10	>4	>4	0.2	>10
18	2	≥40	>4	>4	>4	>4
19	3	≥200	≥200	200	7	>200
20	4	≥40	>10	>10	0.7	>10
21	5	400	150	70	2	>200
22	6	≥10	>10	>10	0.2	>10
23	7	≥100	>40	>40	0.2	>100
24	8	≥40	>10	>10	0.7	>10
25	9	≥40	>10	20	2	>40
26	10	≥40	>10	7	2	>10
27	2	≥100	>100	>40	20	>100
tubercidin		≥1	>0.4	>0.4	>0.1	0.2
(*S*)-DHPA		>400	>400	>400	>70	>70
ribavirin		>400	>400	300	20	>400
carbocyclic 3-deazadenosine		>400	>400	400	2	0.7

[a]Number of CH_2 groups in linker. [b]Required to cause a microscopically detectable alteration of normal cell morphology. [c]Required to reduce virus-induced cytopathogenicity by 50%.

*From Prof. J. W. Lown(ref. 19).

Table 3. Cytotoxicity and antiviral activity of the linked lexitropsins against vaccinia virus in different cell cultures*

		min cytotoxic concn,[b] μg/mL				min inhibitory concn,[c] μg/mL, vaccinia virus					
						PRK			Vero		
cmpd	n[a]	PRK	HeLa	Vero	E_6^{SM}	expt 1	expt 2	HeLa	expt 1	expt 2	E_6^{SM}
16	0	40	≥40	≥40	100	7	7	20	20	20	2
17	1	40	≥40	≥40	≥40	2	2	2	2	2	0.2
18	2	≥100	≥100	≥200	≥200	2	2	2	2	7	0.2
19	3	≥200	≥400	≥400	400	20	20	40	70	150	7
20	4	≥40	≥400	100	100	2	0.7	1	2	7	0.2
21	5	≥200	>200	>200	200	7	7	20	20	20	0.7
22	6	≥40	≥200	>40	≥200	0.7	2	2	7	7	0.07
23	7	≥100	≥200	>200	≥100	7	7	20	40	70	0.7
24	8	≥40	100	≥40	≥100	2	2	20	20	>40	0.2
25	9	≥40	100	100	≥40	2		20	>40		0.2
26	10	≥40	≥40	≥100	≥40	2		2	>40		0.2
27	(2)	≥400	>400	≥400	>400	300	400	>400	>400	>400	70
10	(2)	≥4	≥20	≥4	>4	>4	>4	>10	>4	>4	>4
tubercidin		≥0.4	≥1	≥0.4	4	>0.1	>0.1	0.2	0.02	0.02	0.07
(*S*)-DHPA		>400	>400	>200	>400	100	150	70	20	20	100
ribavirin		>400	≥400	>400	>400	20	7	20	7	20	70
carbocyclic 3-deazaadenosine		>400	>400	>200	>400	2	2	0.7	0.2	0.2	2

[a]Number of CH_2 groups in the linker. [b]Required to cause a microscopically detectable alteration of normal cell morphology. [c]Required to reduce virus-induced cytopathogenecity by 50%.

*From Prof. J. W. Lown(ref. 19).

Table 4. Inhibitory effects of linked lexitropsins on the proliferation of murine leukemia (L1210), murine mammary carcinoma(FM3A), human B-lymphoblast(Raji), and human T-lymphoblast(Molt/4F) cells*

cmpd	n^b	ID_{50},[a] μg/mL			
		L1210	FM3A	Raji	Molt/4F
1 (netropsin)		245 ± 92	321 ± 18	139 ± 63	
2 (distamycin)		27 ± 4.7	31 ± 2.4	24 ± 3.7	
16	0	>100	>100	>100	>100
17	1	28.5 ± 9.7	5.87 ± 2.23	3.39 ± 0.69	2.85 ± 0.64
18	2	>100	47.2 ± 27.8	26.4 ± 2.8	33.8 ± 0.9
19	3	>100	>100	>100	>100
20	4	24.3 ± 8.9	57.8 ± 32.7	13.3 ± 5.6	5.62 ± 0.60
21	5	>100	≥100	11.1 ± 3.2	5.74 ± 1.85
22	6	3.34 ± 0.27	3.15 ± 0.72	2.14 ± 0.80	1.74 ± 0.28
23	7	10.8 ± 6.6	32.0 ± 9.7	4.36 ± 0.89	3.30 ± 0.99
24	8	4.21 ± 1.52	22.1 ± 11.1	3.24 ± 0.44	2.97 ± 0.49
25	2	>100	>100	>100	>100
26	2	>100	4.29 ± 1.12	41.4 ± 8.4	2.85 ± 0.64

[a]50% Inhibitory dose; [b]number of CH_2 units in linker.

*From Prof. J. W. Lown(ref. 19).

IV. B Anti-retroviral Activity

Lexitropsins also displayed marked inhibitory properties against certain enzymes most notably reverse transcriptases. An homologous series of polymethylene linked bis-netropsins exhibited inhibitory activity against Moloney leukemic virus reverse transcriptase in which the activity correlates with the DNA binding constants (Fig.10).[18b]

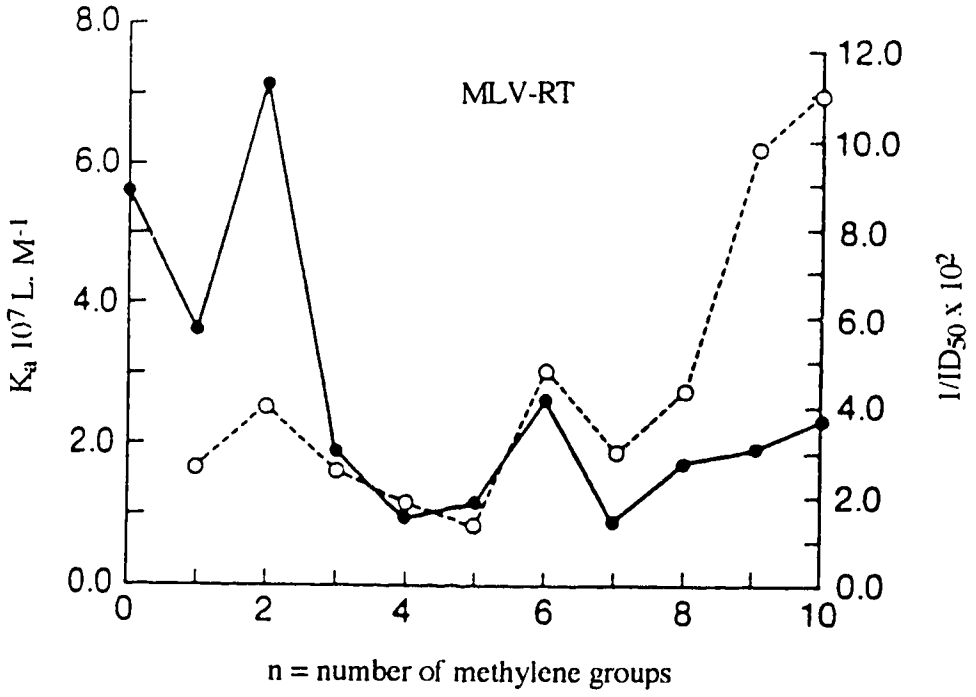


Figure 10 Correlation between DNA binding constants of linked lexitropsins (K_a solid line) and observed inhibitory properties, expressed in reciprocal ID_{50} values, against Moloney leukemia virus reverse transcriptase (From prof. J. W. Lown, ref. 19).

This led to the evaluation of inhibition of HIV-I of these agents in cell culture. Among the thirty-three of five classes of lexitropsins submitted to the NCI-HIV-I screen, ten are assigned active, fifteen moderately active, and the rest inactive.[47] Most of active compounds come from rigid-linked bis-netropsins and distamycins. Among the active compounds, the therapeutic index of one agent exceeded 600 and the undesired cell growth phenomenon did not appear. This result confirms the anti-HIV-I activity proposal for lexitropsins and suggests that further development be undertaken to exploit their chemotherapeutic potential.

IV. C Topoisomerase Inhibition

It has been reported that certain DNA minor groove binders inhibit topoisomerases.[48] Recently, a series of linked bis-netropsins were found to inhibit the activity of isolated topoisomerase II and interfere with the stabilization of cleavable complexes of topoisomerase II and I in cell nuclei.[49] The inhibitory properties increased with increasing number of N-methylpyrrole units for fixed methylene linker length (n=2) and this result is in accord with increased DNA binding. Dimers with linker consisting of 0-4 and 6-9 number of methylene groups were far more inhibitory than netropsin against isolated enzyme in the nuclear system. The dimer with n=5 was inactive while the compound with n=10 inhibited the isolated enzyme only. These results indicated that bidentate binding can significantly enhance anti-topoisomerase activity for methylene linked bis-netropsins, while other factors such as linker length, the number of pyrrole moieties and the nature of the target also contribute to the activities. Another instance of topoisomerase II inhibition by sequence specific minor groove agents is found in certain Hoechst 33258 analogues (Fig. 11).[50] Two binding sites were reported for *Drosophila* topoisomerase II, as underlined in the sequence ACAATG/CGCTCATC (the / sign specifies the enzyme cleavage site).

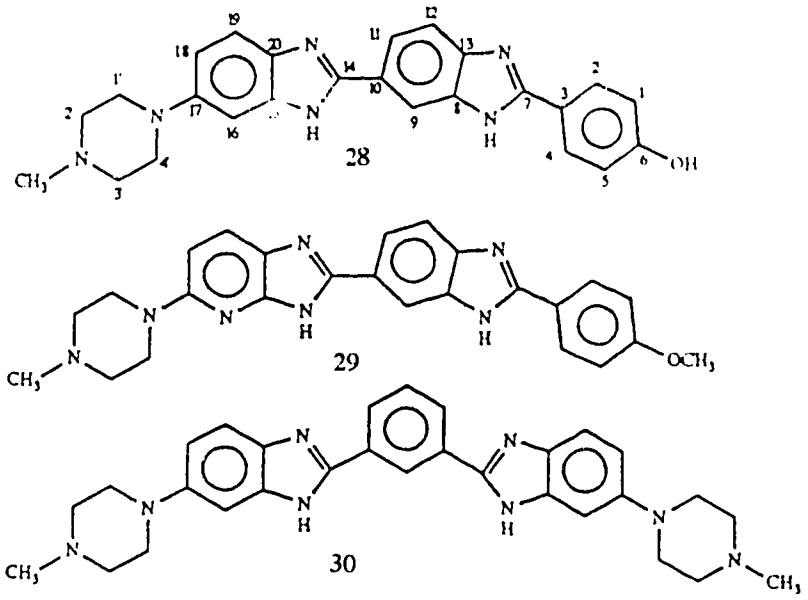


Figure 11 Structures of Hoechst 33258(28) and some new analogues(29), (30).

In conclusion, the topoisomerase inhibition, taken together with the observed inhibitory activity of lexitropsins against cancer and viruses especially vaccinia and HIV-I, indicate the potential for DNA sequence selective interventions in cellular process, and reveal the prospect of development of effective and selective antivirals and anticancer of a novel structural type.

Chapter 2

Synthesis of Head-to-tail Polymethylene-linked Bis-lexitropsins

I Pre-synthetic Analysis

The synthetic approach to the B-DNA minor groove ligands netropsin, distamycin, and lexitropsins is given by their retrosynthetic view as oligopeptides consisting of the non-proteinogenic amino acid 4-amino-1-methylpyrrole-2-carboxylic acid (H_2N-Pyr-OH), and C- and N-terminal end groups. The N-terminal end group for the lexitropsins, as well as distamycin, is the formyl group; while for netropsin, it is guanidinium acetic acid. The C-terminal end group is usually the ß-amino-propionamidinium group. As indicated previously, the major function of the latter is to bear a positive charge to provide electrostatic attraction to the DNA. In addition, hydrophobic interactions exits between the ligand and the floor of the minor groove, and bifurcated hydrogen bonds are formed from amide hydrogens to A-N_3 and T-O_2.

A number of early total synthesis of these natural oligopeptides have been reported.[51-56] All these synthetic methodologies have utilized 1-methyl-4-nitropyrrole-2-carboxylic acid 31 as a starting material. The acid 31 was originally prepared by a five-step route, starting from furan-2-carboxylic acid.[53] The disadvantages of the earlier method have been circumvented by a simplified procedure starting from 1-methylpyrrole-2-carboxylic acid [54] or its esters.[55] The nitration method was improved for the preparation of 31, but the yield was still not satisfactory.[56] A novel approach to the synthesis of the oligopeptides was recently reported in which the basic unit could be obtained on a large scale and the peptide formation process was simplified.[57] The basic building block in this oligopeptide synthesis is 1-methyl-4-nitro-2-trichloroacetylpyrrole 32.

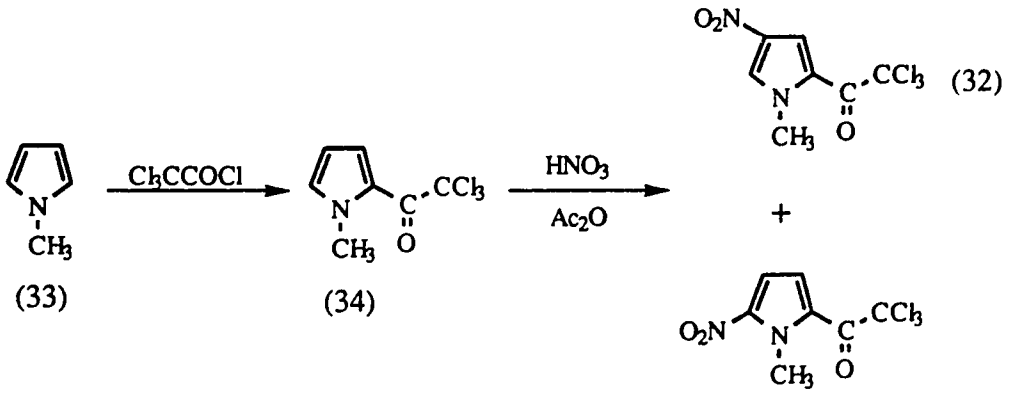
In this chapter I describe my approach to the "Head" to "Tail" flexible polymethylene-linked bis-lexitropsins HCO-Pyr_2-A_xa-Pyr_2-PAH.

II Description of the Experiments

II.A Preparation of the Basic Building Unit

The starting material used in the preparation was 1-methyl-2-trichloroacetylpyrrole 34 which could be easily prepared from commercially available N-methyl-pyrrole 33, by a modification of Rapoport's method, in 83% yield. Nitration of 34 gave a mixture of 4-nitro and 5-nitro derivatives,[57] which was similar to the result of the nitration of 2-trichloroacetylpyrrole,[59] but the desired 4-nitro isomer 32 was the predominant product and could be separated by simple work-up processes. When 34 was nitrated with 90% nitric acid-acetic anhydride mixture at -40 °C, the desired 1-methyl-4-nitro-2-trichloroacetylpyrrole 32 was precipitated from the reaction mixture by adding isopropyl alcohol at -20 °C. After collection and washing with isopropyl alcohol, pure 4-nitro isomer 32 was obtained in 53% (Scheme 1). Compound 32, a white solid, can be stored at room

Scheme I



temperature for months without any detectable change, making it a convenient and versatile

precursor for synthesis of various oligomers. One problem associated with this reagent is that it causes skin allergy. It was known that the trichloroacetyl group can be converted to the carboxylic acid, esters, or aliphatic amides with facility.[60] Thus a hydrolysis reaction was carried out in the presence of base to give the corresponding acid in quantitative yield, although compound 32 was very stable in hot aqueous solution.

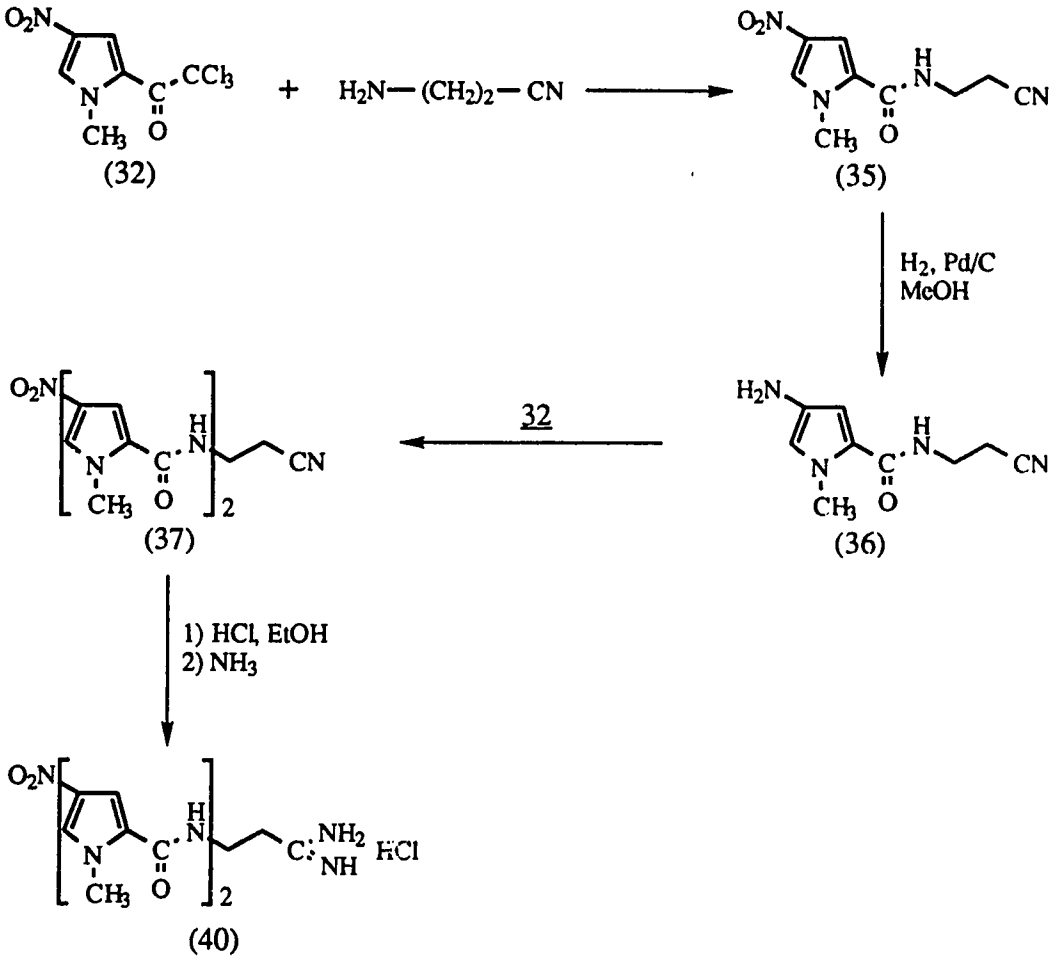
II. B Set-up of the End Group (O_2N)-Pyr-Pyr-PAH

The synthesis of (O_2N)-Pyr-PN 35 was described by Shibuya's group,[57] wherein (O_2N)-Pyr-CCl_3 32 was used as the reactive substitute for (O_2N)-Pyr-COOH 31(Scheme II). The condensation reaction between 3-amino-propionitrile and compound 32 was carried out at room temperature and no precaution in terms of air or light or moisture needs to be taken. Moreover, this procedure avoids the use of relatively laborious work-up operations for the usual peptide synthesis and provides an excellent yield on a large scale provided that the amine is reactive enough.

The coupling of (O_2N)-Pyr-PN with (O_2N)-Pyr-CCl_3 to form (O_2N)-Pyr_2-PN 37 was also described by Shibuya's group (Scheme II).[57] Performing the experiment under a slightly modified conditions gave the desired product in 74% yield compared with 90% reported. It was found that the reaction should be carried out with the exclusion of air and light and run at relatively low temperature or the amine 36 would polymerize to a dark brown gel-like residue. Complete removal of solvent methanol before the coupling reaction was also essential, since 1-methyl-4-nitro-2-trichloroacetylpyrrole reacted easily with methanol to form an ester. This was done by dissolving the amine residue in DMF, then removing half of the solvent in high vacuum.

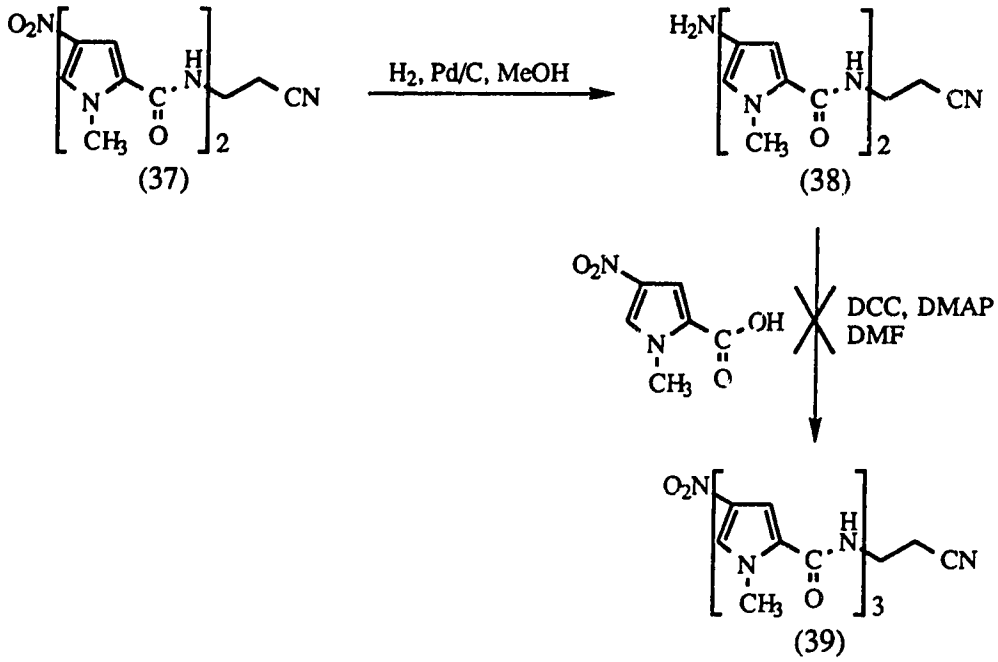
After the reduction of NO_2 group of compound 37, the resulting amine 38 was coupled with 1-methyl-4-nitropyrrole-2-carboxylic acid in the presence of DCC and DMAP.

Scheme II



However, the expected product 39 was not obtained (Scheme III). It is probable that the amine 38 is very unstable at the reaction temperature (23 °C) and deteriorates rapidly. In addition, the preparation of the amidinium function from the nitrile group by the Pinner reaction requires rather harsh conditions (HCl gas in ethanol),[61] which may cause problems if the reaction is carried out at the latter stage. Therefore, compound 37 was converted to the corresponding amidinium chloride by a modified two-step Pinner reaction in excellent yield.

Scheme III



II.C Set-up of the Central Portion (O_2N)-Pyr_2-A_xa

Since amino acids exist in their zwitterionic forms under neutral conditions, the reactivities of the C- and the N-terminals have to be inverted to make the N-terminus nucleophilic or the C-terminus electrophilic:

$$H_2N\text{-}(CH_2)x\text{-}COOH \rightleftharpoons H_3{}^+N\text{-}(CH_2)_x\text{-}COO^-$$

One general way is to protect the N-terminus with Boc-group and activate the carboxylic group. Some examples of carboxylic activations for peptide/amide formation in the literature [62] are: (1) acid chloride via acid and $SOCl_2$, (2) acid chloride via acid and $(NPCl_2)_3$,[63] (3) acid fluoride via acid and "cyanuric fluoride" (2,4,6-trifluoro-1,3,5,-triazine),[64] (4) improvements of DCC and other carbodiimide coupling methods via active-

ester intermediates, e.g. HOBt ester,[65] PFP (pentafluorophenol) esters,[66] and ECDI in buffered aqueous solutions,[67,68] (5) N-hydroxy esters from acids and tetramethyluronium salts, as O-(N-succinimido)-N,N,N',N'-tetramethyluroniumtetrafluoroborate,[69] and (6) the simple addition of N-hydroxy compounds like N-hydroxy succinimide (HSU) and 1-hydroxybenzotriazole (HOBt) accelerates the coupling reaction with DCC, and suppresses racemization.[70]

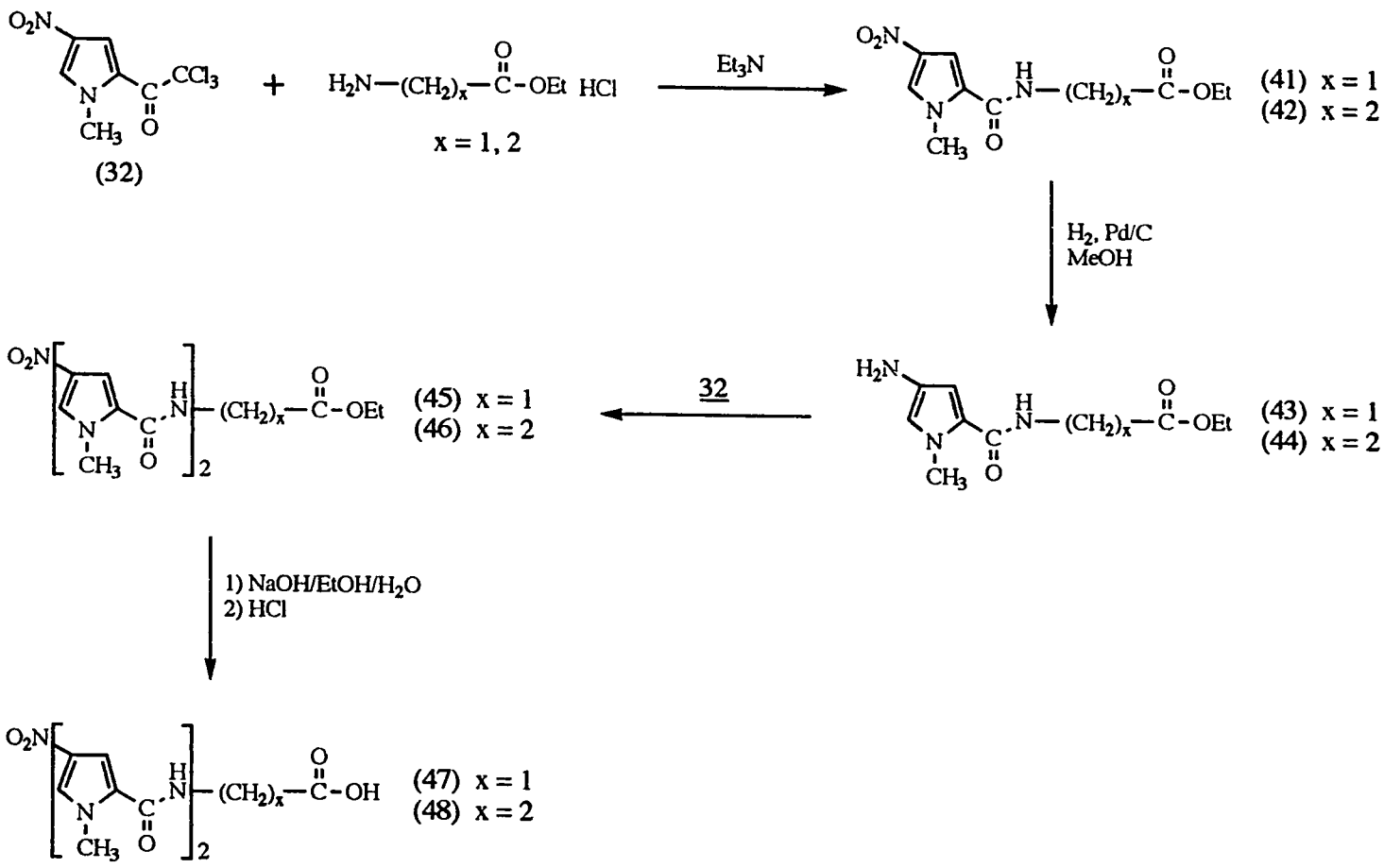
An alternative way to form peptide/amide bond in amino acid synthesis is to protect the carboxyl group. A number of carbonyl protection methods are[71]: (1) formation of methyl or ethyl ester via acid and alcohol in the presence of acid and base catalyst, (2) formation of trimethylsilyl ethyl ester,[72] (3) transformation of acyl group to its tri-n-butyltin esters via an acid and tri-n-butyltin oxide,[73] or (4) condensation reaction in the presence of a base without protecting the carboxyl group. Depending on whether or not the starting material is commercially available, I chose two different methods to prepare the middle part (O_2N)-Pyr_2-A_xa.

II.C.a Preparation of the Central Portion via Ethyl Ester Formation

Glycine and alanine ethyl ester hydrochlorides are commercially available material in large quantity. Condensation between these esters with 1-methyl-4-nitro-2-trichloroacetyl-pyrrole 32 gave the corresponding products in excellent yield with effortless purification. Then the nitro group of 41 was reduced to the amine which was then condensed with 32 to give the dipeptide 45. Saponification of 45 at 70°C yielded the corresponding acid 47 in 47% overall yield. Similarly, the dipeptide acid 48 was prepared in 62% overall yield(Scheme IV).

II.C.b Preparation of the Central Portions via Tri-n-butyltin Ester Formation

Scheme IV

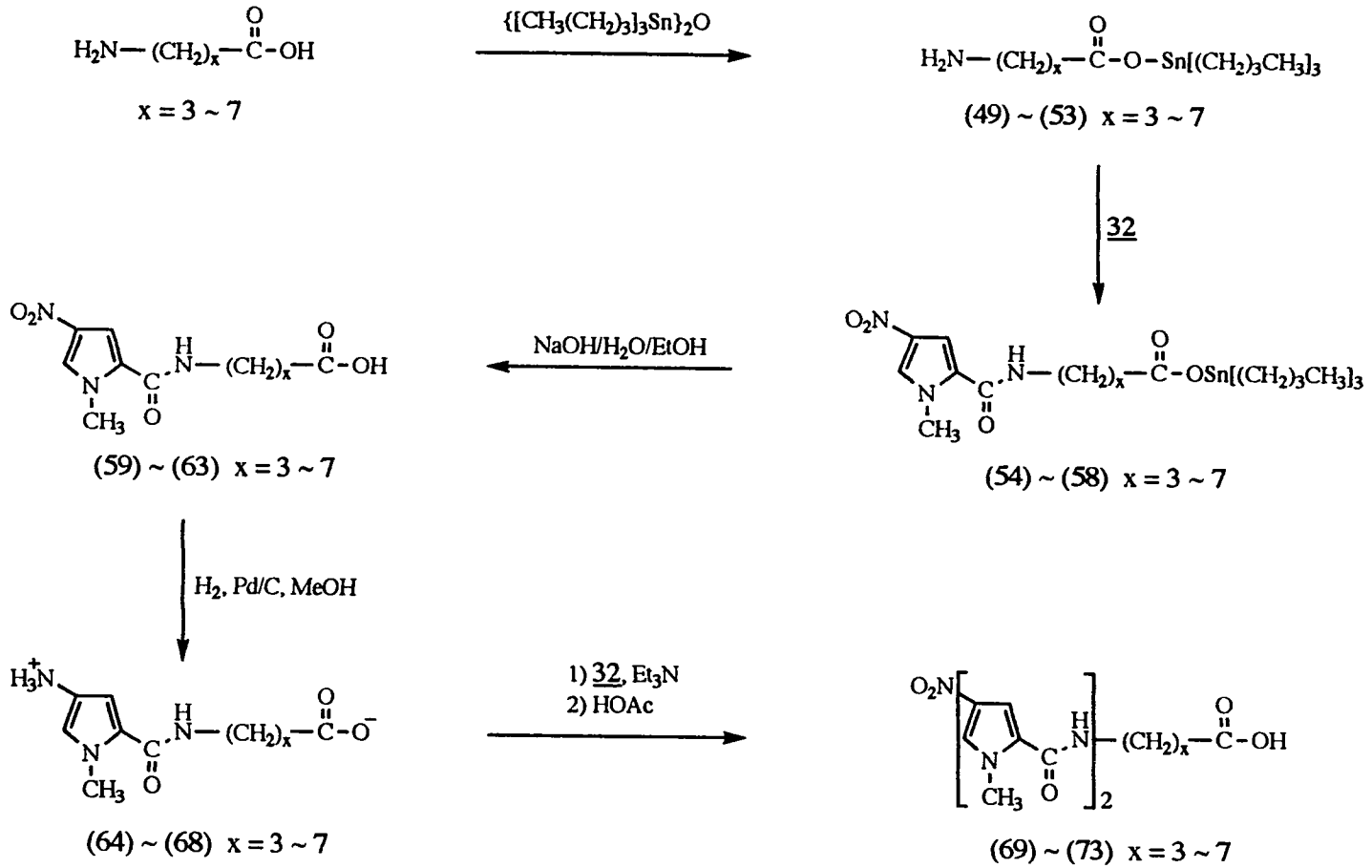

(32)
x = 1, 2
Et3N
(41) x = 1
(42) x = 2
H2, Pd/C
MeOH
(43) x = 1
(44) x = 2
32
(45) x = 1
(46) x = 2
1) NaOH/EtOH/H2O
2) HCl
(47) x = 1
(48) x = 2

Amino acids $NH_2(CH_2)_xCOOH$ (X=3 to 7) are purchased from Aldrich. It was found that the most convenient access to the (O_2N)-Pyr-A_xa (x=3 to 7) was to transform the amino acids to their tri-n-butyltin esters by refluxing a suspension of the ω-amino acids with one half equivalent of bis-(tri-n-butyltin)oxide (Caution! Avoid skin contact, as it is readily taken up by skin and interferes with the lymph system) in benzene in a Dean-Stark water separator until the suspension had turned into a clear and colorless solution. The use of benzene instead of toluene decreases the reaction temperature, so that the formation of lactams is suppressed in favour of the 4-amino-butyric as well as 5-amino-valeric esters.[74] These highly moisture-sensitive butyltin esters were concentrated to small volumes and then treated with (O_2N)-Pyr-CCl_3 with the exclusion of moisture. Cleavage of the tin esters by contact with dilute NaOH (extraction of the benzene solution with NaOH) and acidification of the aqueous solution with concentrated HCl to pH 3 gave analytically pure crystals ((O_2N)-Pyr-NH-$(CH_2)_x$-COOH, x=3 to 7) in about 80% yields (Scheme V).

Hydrogenation of compounds 59-63 was carried out at room temperature and in H_2(1 atm) atmosphere, using MeOH as a solvent. MeOH was found to be a good solvent for both the nitro compound and the amine, and it is a proton source and can be easily removed in vacuo. As mentioned earlier, since it is crucial that MeOH must be removed completely before the coupling partner (O_2N)-Pyr-CCl_3 is added and the concentrated amine is very unstable in the air, the hydrogenation was performed in a MeOH/DMF (1/1) mixture solvent, and after being filtered from the Pd/C catalyst, the solvent was removed by *rotavapor* then in high vacuum until 1/3 of the original volume. The coupling of amines with (O_2N)-Pyr-CCl_3 was performed at low temperature in argon to give (O_2N)-Pyr-A_xa (x=3 to 7) in approximately 75% yield (Scheme V).

II. D Coupling of the Central Part (O_2N)-Pyr_2-NH-(CH$_2$)xCOOH and the Right End Part (O_2N)-Pyr_2-PAH

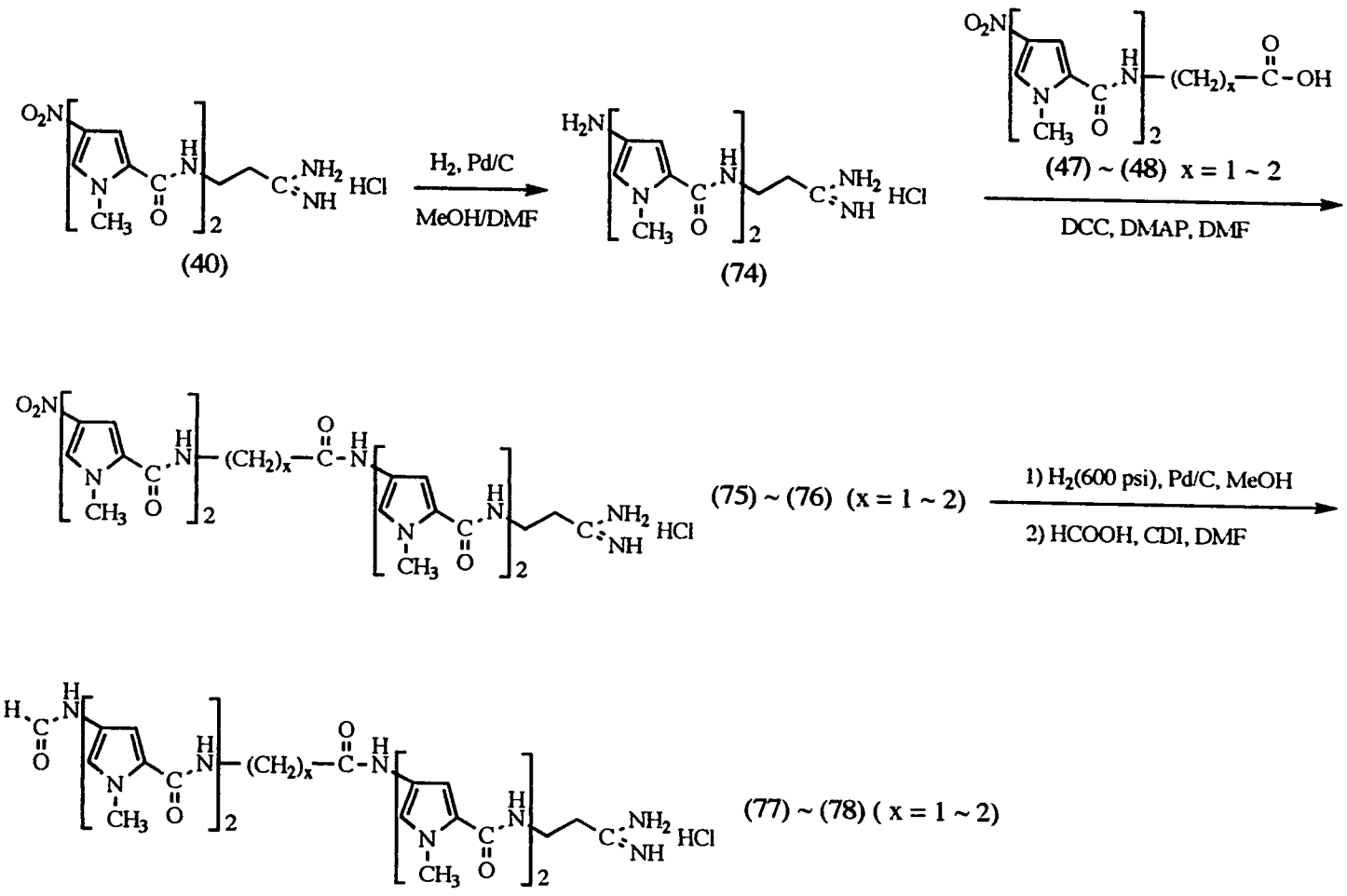
Scheme V


{[CH3(CH2)3]3Sn}2O
x = 3 ~ 7
(49) ~ (53) x = 3 ~ 7
32
NaOH/H2O/EtOH
(54) ~ (58) x = 3 ~ 7
(59) ~ (63) x = 3 ~ 7
H2, Pd/C, MeOH
1) 32, Et3N
2) HOAc
(64) ~ (68) x = 3 ~ 7
(69) ~ (73) x = 3 ~ 7

Amidine ((O_2N)-Pyr_2-PAH), a fine yellowish solid, is not soluble in any of the solvents tested except sparingly soluble in DMF. However, reduction of a nitro-pyrrole to an amino-pyrrole increases the solubility considerably so that the hydrogenated compound 74 is easily soluble in MeOH(Scheme VI). Hydrogenation was performed on Paar Shaker at 70 psi for 4 hours, using 1 equivalent of Pd/C catalyst. Coupling of the amine residue with the central part (O_2N)-Pyr_2-NH-CH_2-COOH in the presence of DCC failed to give the desired lexitropsin precursor 75, but yielded a dark-brown residue. However, successful coupling was achieved in the presence of 4-dimethylaminopyridine (DMAP), which is believed to act as a catalyst in the reaction, even though the yield needed to be improved. Lexitropsin precursor 76 was synthesized in a similar manner.

The purification of compound 75 and 76 imposed a problem since they were so polar that they could hardly be washed out from the silica column by MeOH/HOAc(9/1). Later it was found that the compound could be separated by the reversed phase HPLC method, using MeOH/H_2O as an eluent and ammonium acetate as a buffer (to increase the ionic strength of the eluent). The ammonium salt could be removed by repeated freeze-drying for several times. On a semi-preparative scale, each run took about 4 hours and afforded approximately 5 milligrams of pure lexitropsin precursor. The efficiency of separation can be increased if the pretreated sample is injected. The pretreatment of samples is a multi-step dissolving-extraction process and is described as follows: the solid mixture is washed with chloroform a few times to remove most of the urea, then dissolved in the minimum amount of methanol and extracted with ethyl acetate. The process is repeated until almost all the desired component has been extracted. Reduction of nitro group of 75 was performed in a Hydrogen Bomb (600 psi) over a period of 2 hours with vigorous shaking (to disperse the catalyst in the solvent). Formylation of the amine residue with a prepared solution of formic acid and 1,1'-carbonyldiimidazole (CDI) gave a mixture which again was separated

Scheme VI

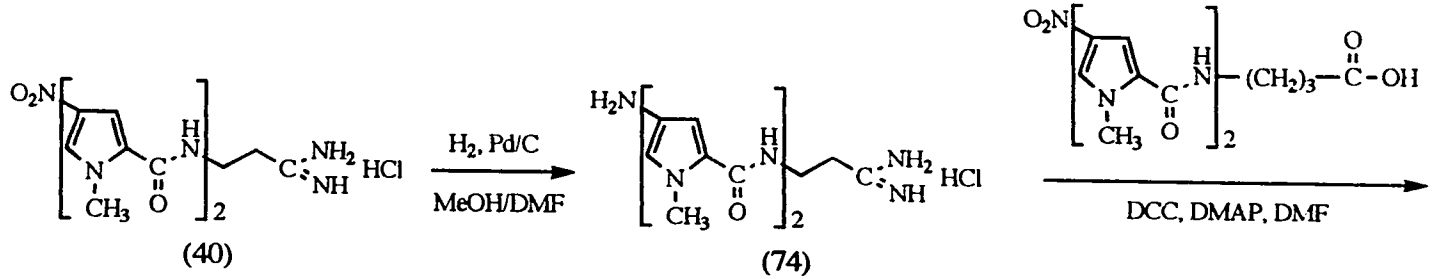

(40)
H2, Pd/C
MeOH/DMF
(74)
(47) ~ (48) x = 1 ~ 2
DCC, DMAP, DMF
(75) ~ (76) (x = 1 ~ 2)
1) H2(600 psi), Pd/C, MeOH
2) HCOOH, CDI, DMF
(77) ~ (78) (x = 1 ~ 2)

by reversed phase HPLC method to give pure lexitropsin 77. Similarly, the lexitropsin 78 was prepared (Scheme VI).

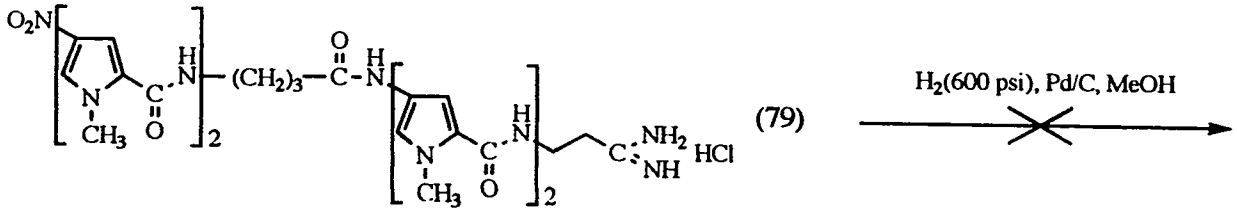
II. E Formylation of the Central Portion (O_2N)-Pyr_2-NH-(CH_2)x-COOH (x=3 to 7) and Coupling with the Right End Part (O_2N)-Pyr_2-PAH

Even though the coupling reaction of the central portion (O_2N)-Pyr_2-NH-$(CH_2)_x$-COOH (x=3) with the reduced right end moiety (H_2N)-Pyr_2-PAH proceeded, the nitro group of the lexitropsin precursor 79 could not be reduced to the amino group under the high pressure of H_2 (600 psi) with vigorous shaking (Scheme VII). An alternative way is to perform the formylation of the central part before the coupling reaction. Thus treatment of the reduced form of the central portion (H_2N)-Pyr_2-NH-$(CH_2)_3$-COOH with a prepared solution of HCOOH and CDI in the presence of triethylamine at low temperature (-40 °C) gave the formylated coupling precursor anion which was protonated by adding one equivalent of acetic acid. After being purified on a flash silica column, this coupling precursor was allowed to react with the reduced right end group (H_2N)-Pyr_2-PAH in the presence of DCC and DMAP to give a crude lexitropsin 86. The latter was purified by the reversed phase HPLC method. Similarly, lexitropsins 87 to 90 were synthesized(Scheme VIII).

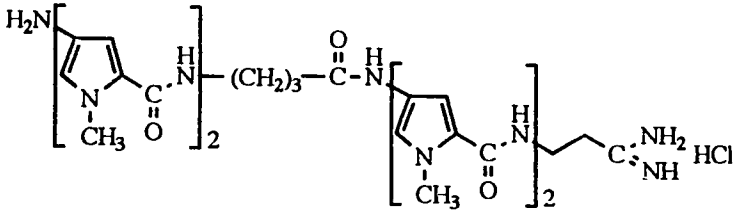
Scheme VII



(40) (74)

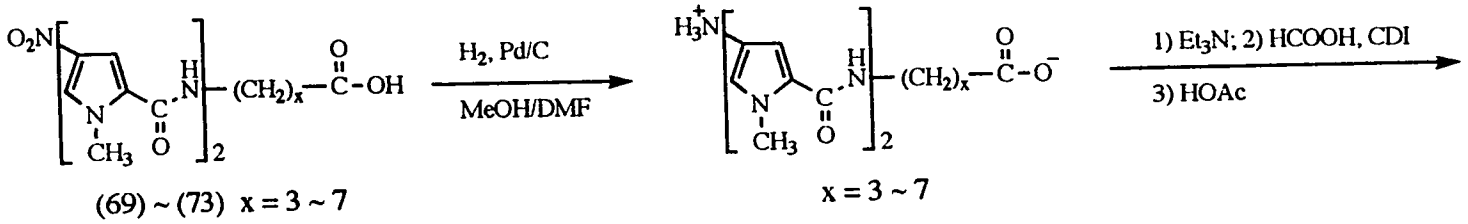


(79)



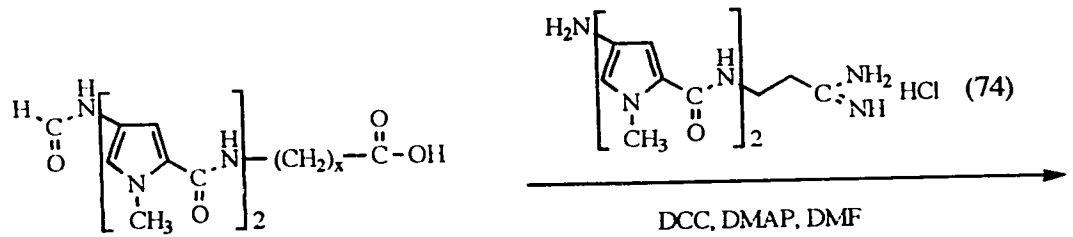
(80)

Scheme VIII

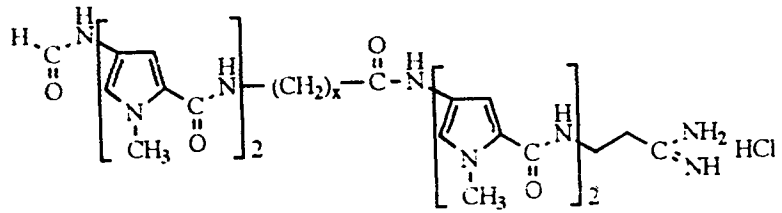


(69) ~ (73) x = 3 ~ 7

x = 3 ~ 7



(81) ~ (85) x = 3 ~ 7



(86) ~ (90) (x = 3 ~7)

III Experimental

General

Melting points were measured on an Electrothermal melting point apparatus and are uncorrected. The IR spectra were recorded on a Nicolet 7199FT spectrometer. The ^{1}H-NMR spectra were recorded on Bruker WH-200, AM-300, and WH-400 spectrometers. Fast atom bombardment mass spectra(FAB-MS) using glycerol as the matrix were determined on Associate Electrical Industries(AEI) MS-9 and MS-50 focusing high resolution mass spectrometers. Elemental combustion analyses were performed on Carlo Erba Instruments' EA1108-Elemental analyzer. Reversed-phase HPLC was performed on Waters Associates liquid chromatography using Waters' C4 semipreparative column. Buffer salt(used in the eluent) was removed on Labconco Freeze Dryer 4.5.

Materials

Kiesselgel 60 (230-400 mesh) of E. Merck was used for all flash chromatography, and precoated silica gel 60F-254 sheets of E. Merck were used for TLC and the spots were visualized under UV light. All compounds obtained commercially were used without further purification. Ethanol and methanol were freshly distilled from Mg turnings. THF was distilled from Na/benzophenone under an atmosphere of argon. Ether was dried over Na. Methylene chloride was distilled from P_2O_5 and stored over molecular sieves (3Å). Et_3N and Hunig's base was stored over KOH pellets. DMF was distilled over CaH_2 in the reduced pressure and stored over molecular sieves(3Å).

1-Methyl-2-trichloroacetylpyrrole (34). To a solution of trichloroacetyl chloride (45.5 ml, 405 mmol) in CH_2Cl_2 (240 ml) was added a solution of N-methylpyrrole (35.5 ml, 400 mmol) in CH_2Cl_2 (160 ml) over a period of 3 h. During this time, the solution was stirred vigorously and argon was swept through to remove HCl as it

was formed. The solution was stirred overnight and solvent evaporated. The brown residue was dissolved in $CHCl_3$ and filtered through a short column of silica gel. Evaporation of solvent gave light yellow crystals (74.6 g, 82.3% yield); m.p. 64-66 °C (lit. m.p.[57] 64-65°C).

1-Methyl-4-nitro-2-trichloroacetylpyrrole (32). To a suspension of 32 (52.5 g, 232 mmol) in acetic anhydride (300 ml) at -40 °C, 20 ml of HNO_3 (90%) was added over a period of 45 min. and then allowed to warm up to -20 °C. Isopropyl alcohol (300 ml) was added and the white crystals that resulted were collected, washed with cold isopropyl alcohol, dried under reduced pressure to give 32 as white crystals (33.04 g, 52.5% yield); Mp 134-136 °C (lit.[57]m.p. 135-140 °C). Ir($CHCl_3$) 1690, 1520, 1310 cm^{-1}. Nmr(DMSO-d_6) δ 4.01(3H, s), 7.80(1H, d, J=1.8 Hz), 8.56(1H, d, J=1.8 Hz). Anal. calcd for $C_7H_5Cl_3N_2O_3$(%): C, 30.97; H, 1.85; N, 10.32. Found: C, 30.95; H, 1.80; N, 10.05. The filtrate was concentrated and dissolved in 120 ml of ethyl acetate, washed with Na_2CO_3 and brine, then dried over anhydrous $MgSO_4$. The mixture was filtered and the solvent evaporated. Ethyl ether was added and the resultant crystal collected, washed with ethyl ether to give additional 32 (14.02 g, 22.26% yield).

1-Methyl-4-nitropyrrole-2-carboxamidopropionitrile (35). A solution of 3-aminopropionitrile(4.10 g, 58.5 mmol) in 5 ml of THF was added dropwise to a stirred solution of 32 (14.47 g, 53.2 mmol) in 90 ml of THF at 0 °C. The reaction mixture was warmed up to r.t. and stirring was continued for 22 h. The solvent was removed in vacuo and the residual solid was recrystallized from ethanol to give pale yellow needles (10.73 g, 83% yield), Mp 140-142 °C (lit.[57] mp 135 °C). Ir ($CHCl_3$) 1660, 1550, 1520, 1500, 1420, 1310 cm^{-1}. ^1H Nmr (DMSO-d_6) δ 2.73 (2H, t, J=6.4 Hz), 3.43 (2H, m), 3.91 (3H, s), 7.45 (1H, d, J=1.8 Hz), 8.16 (1H, d, J=1.8 Hz), 8.76 (1H, t, J=5.2 Hz). FAB-MS, m/z 223 (MH^+, 24%). Anal. calcd for $C_9H_{10}N_4O_3$: C, 48.65; H, 4.54; N, 25.21. Found: C, 48.58; H, 4.39; N, 24.99.

1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamidopropionitrile (37). A suspension of 10% Pd/C (1g) in a solution of 35 (2.30 g, 10.4 mmol) in MeOH (50 ml) was stirred for 4 h in H_2 (1 atm) at r.t., then filtered. The residual catalyst was washed thoroughly with MeOH and DMF and the combined filtrate and washings were concentrated in vacuo to give the crude amine residue. To a solution of this amine in DMF (5 ml) cooled down to 0 °C, 32 (2.81 g, 10.4 mmol) in THF (5 ml) was added dropwise, then the solution was brought up to r.t. Stirring was continued at r.t. for 16 h. Solvent was removed and EtOH (30 ml) was added, the solution was boiled, then cooled, and the resultant yellow crystal collected (2.65 g, 74.4% yield). Mp 244-245 °C (lit. m.p. 254-255 °C). Ir (KBr) 3400, 3130, 2240, 1662, 1649, 1559, 1530, 1495, 1300 cm^{-1}. Nmr (DMSO-d_6) δ 2.72 (2H, t, J = 6.6 Hz), 3.39 (2H, m), 3.82 (3H, s), 3.94 (3H, s), 6.92 (1H, d, J = 1.8 Hz), 7.23 (1H, d, J =1.8 Hz), 7.57 (1H, d, J = 1.8 Hz), 8.18 (1H, d, J = 1.8 Hz), 8.40 (1H, t, J = 5.6 Hz), 10.26 (1H, s). FAB-MS: m/z 345 (MH^+, 4.7%). Anal. calcd for $C_{15}H_{16}N_6O_4$ (%): C, 51.80; H, 4.63; N, 24.16. Found: C, 52.04; H, 4.67; N, 23.78.

1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamidopropionamidine hydrochloride (40). A suspension of 37 (1.50 g, 4.36 mmol) in anhydrous ethanol (50 ml) was treated with HCl gas at 0 °C until the solution became clear. The mixture was stirred at r.t. for 2 h then the solvent was removed in vacuo. The solid residue was twice washed with anhydrous ether and dried . Anhydrous EtOH (30 ml) was added and NH_3 gas was condensed into the vessel. After being stirred at r.t. for 16 h, the solvent was removed under reduced pressure. Ethanol was added, the resultant yellow powder collected (1.69 g, 98% yield). Mp 246 °C. Ir (KBr) 3130, 3100, 1640, 1590, 1500, 1420, 1315, 1255, 1120 cm^{-1}. Nmr (DMSO-d_6) δ 2.63 (2H, t, J = 6.2 Hz), 3.51(2H, m), 3.84 (3H, s), 3.97 (3H, s), 6.94 (1H, d, J = 1.8 Hz), 7.52 (1H, d, J = 1.8 Hz), 7.61 (1H, d, J = 1.8 Hz), 8.18 (1H, d, J = 1.8 Hz), 8.29 (1H, t, J = 6.5

Hz), 8.65 (2H, s), 8.98 (2H, s), 10.29 (1H, s). Calcd mass for $C_{15}H_2N_7O_4$: 362.1577. Found via exact mass FAB: 362.1577 (2.4%).

Ethyl 1-methyl-4-nitropyrrole-2-carboxamidoacetate (41). A suspension of glycine ethyl ester hydrochloride (1.41 g, 10 mmol) and Hunig's base (1.9 ml, 11 mmol) in THF (5 ml) was stirred at r.t. for 2 h. To this mixture, a solution of 32 (2.72 g, 10 mmol) in THF (15 ml) was added. Stirring was continued at r.t. for 2 h then at 50 °C for 16 h. The solvent was removed in vacuo, water was added and the resultant solid collected (2.18 g, 85.3% yield). A small amount of this solid was recrystallized from H_2O for analytical purposes. Mp 114-115 °C. Ir (KBr) 3290, 3120, 1730, 1630, 1560, 1530, 1500, 1310, 1210, 760 cm^{-1}. Nmr (DMSO-d_6) δ 1.26(3H, t, J = 6.6 Hz), 3.96 (3H, s), 3.99 (1H, d, J=5.9 Hz), 4.18 (2H, q, J=7.0 Hz), 7.56 (1H, d, J=1.9 Hz), 8.96 (1H, t, J=5.9 Hz). FAB-MS 256 (MH^+, 64%). Anal. Calcd for $C_{10}H_{13}N_3O_5$: C, 46.11; H, 5.02; N, 16.13. Found: C, 46.46; H, 4.91; N, 16.14.

Ethyl 3-(1-methyl-4-nitropyrrole2-carboxamido)propionate (42). Synthesis of this compound was based on the method of preparing 41. Yield 97%. Mp 138-140 °C. Ir (KBr) 3350, 3120, 1710, 1660, 1550, 1310, 1220, 740 cm^{-1}. Nmr (DMSO-d_6) δ 1.21 (3H, t, J = 7.0 Hz), 2.54 (2H, t, J = 7.0 Hz), 3.42 (2H, m), 3.90 (3H, s), 4.07 (2H, q, J = 7.0 Hz), 7.41 (1H, d, J = 1.8 Hz), 8.13 (1H, d, J = 1.8 Hz), 8.49 (1H, t, J = 5.5 Hz). FAB-MS 270 (MH^+, 45%). Anal. calcd for $C_{11}H_{15}N_3O_5$: C, 49.07; H, 5.62; N, 15.61. Found: C, 49, 15; H, 5.62; N, 15.50.

Ethyl 1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamidoacetate (45). A suspension of 10% Pd/C (314 mg) and 41 (757 mg, 2.97 mmol) in MeOH was stirred in H_2 (1 atm) at r.t. for 2 h, then filtered. The catalyst was washed thoroughly with MeOH and the combined filtrate and washings was concentrated in vacuo. DMF (2 ml) was added and evaporated. To a solution of the amine residue in 4 ml of DMF at -20 °C, 32 (805 mg, 2.97 mmol) was added portionwise. The

mixture was stirred at 0 °C for 2 h then at r.t. for 18 h. The solvent was removed, ethanol added, and the resultant crystal collected. Recrystallization gave the analytically pure crystals (0.746 g, 67% yield). Mp 243-244 °C. Ir (KBr) 3400, 3320, 3220, 1740, 1660, 1640, 1570, 1540, 1490, 1310, 1210, 1120, 810 cm^{-1}. Nmr (DMSO-d_6) δ 1.20 (3H, t, J = 7.0 Hz), 3.80 (3H, s), 3.89 (2H, d, J = 6.0 Hz), 3.94 (3H, s), 4.11(2H, q, J = 7.0 Hz), 6.92 (1H, d, J =1.8 Hz), 7.27 (1H, d, J = 1.8 Hz), 7.57 (1H, d, J = 1.8 Hz), 8.18 (1H, d, J = 1.8 Hz), 8.47 (1H, t, J = 5.5 Hz), 10.28 (1H, s). FAB-MS 378 (MH^+, 1.5%). Anal. calcd. for $C_{16}H_{19}N_5O_6$: C, 50.17; H, 4.99; N, 18.27. Found: C, 50.49; H, 4.84; N,18.17.

Ethyl 3-[1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]propionate (46). The synthetic procedure of this compound was similar to that of 45. Yield 66%. Mp 208-209 °C. Ir (KBr) 3400, 3370, 3110, 1715, 1640, 1520, 1500, 1310, 1250, 750 cm^{-1}. Nmr (DMSO-d_6) δ 1.17 (3H, t, J = 7.0), 2.52 (2H, t, J = 7.0), 3.39 (2H, m), 3.79 (3H, s), 3.90 (3H, s), 4.05 (2H, q, J = 7.0), 6.84 (1H, d, J = 1.8), 7.21 (1H, d, J = 7.0), 7.60 (1H, d, J = 1.8), 8.12 (1H, t, J = 5.6), 8.17 (1H, d, J = 1.8), 10.30 (1H, s). FAB-MS 392 (MH^+, 23%). Anal. calcd. for $C_{17}H_{27}N_5O_6$: C, 51.01; H, 5.38; N, 17.80. Found: C, 52.01; H, 5.38; N, 17.53.

1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamidoacetic acid (47). A suspension of 45 (0.746 g, 1.98 mmol) and NaOH (0.087 g, 2.18 mmol) in 5 ml of EtOH and 5 ml of H_2O was stirred at 70 °C until all solid dissolved. The hot solution was acidified with concentrated HCl to pH 3, then EtOH was removed in vacuo. The yellow precipitate was filtered, washed with H_2O and dried under vacuum to give pure solid (0.57 g, 82% yield). Mp 280 °C (dec.). Ir (KBr) 3420, 3120, 1710, 1640, 1530, 1500, 1310, 1190, 1120 cm^{-1}. Nmr (DMSO-d_6) δ 3.81 (3H, s), 3.82 (2H, d, J = 5.8), 3.95 (3H, s), 6.90 (1H, d, J = 1.9), 7.27 (1H, d, J = 1.9), 7.58 (1H, d, J = 1.9), 8.17 (1H, d, J = 1.9), 8.36 (1H, t, J = 5.8), 10.26(1H, s), 12.48 (1H, s).

FAB-MS 350 (MH^+, 2.8%). Anal. calcd. for $C_{14}H_{15}N_5O_6$: C, 46.22; H, 4.16; N, 19.24. Found: C, 46.39; H, 4.29; N, 18.87.

3-[1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]propionic acid (48) The procedure of preparing this compound is similar to that of 47. Yield 96%. Mp 254 °C (dec.). Ir (KBr) 3300, 3112, 1710, 1640, 1630, 1520, 1500, 1310, 1250 cm^{-1}. Nmr (DMSO-d_6) δ 2.46 (2H, t, J = 7.0), 3.36 (2H, m), 3.80 (3H, s), 3.95 (3H, s), 6.83 (1H, d, J = 1.9), 7.20 (1H, d, J = 1.9), 7.56 (1H, d, J = 1.9), 8.09 (1H, t, J = 7.0), 8.18 (1H, d, J = 1.9), 10.24 (1H, s), 12.20 (1H, s). FAB-MS 364 (MH^+, 0.81%). Anal. calcd. for $C_{15}H_{17}N_5O_6$: C, 47.36; H, 4.51; N, 18.41. Found: C, 47.18; H, 4.65; N, 18.21.

4-(1-Methyl-4-nitropyrrole-2-carboxamido)butyric acid (59). To a suspension of 4-amiobutyric acid (1.03 g, 10.0 mmol) in 120 ml of benzene, bis-tri-n-butyltin oxide (3.09 g, 5.0 mmol) was added. The mixture was distilled azetropically on a Dean-Stark water separator in a preheated oil bath (90 °C) until the suspension had turned into a clear colorless solution. Then the solution was cooled to 30 °C and concentrated to a small volume. To this residue, 2.58 g (9.5 mmol) of (O_2N)-Pyr-CCl_3 was added, and the stirring was continued at r.t. for 18 h. The mixture was extracted with 15% NaOH solution three times, and the extract was washed with ether. Acidification of the aqueous solution with concentrated HCl gave pale yellow crystals (2.00 g, 82% yield). Mp 155-156 °C. Ir (KBr) 3330, 3220, 3120, 1750, 1660, 1560, 1530, 1310 cm^{-1}. Nmr (CD_3OD) δ 1.87 (2H, q, J = 7.0), 2.37 (2H, t, J = 7.5), 3.34 (2H, t, J = 7.0), 3.94 (3H, s), 7.28 (1H, d, J = 1.8), 7.82 (1H, d, J = 1.8). FAB-MS 256 (MH^+, 23%). Anal. calcd. for $C_{10}H_{13}N_3O_5$: C, 46.59; H, 5.08; N, 16.30. Found: C, 46.56; H, 5.04; N, 16.08.

5-(1-Methyl-4-nitropyrrole-2-carboxamido)valeric acid (60). The procedure is similar to that above. Yield 81%. Mp 131-132 °C. Ir (KBr) 2920, 1730,

1620, 1530, 1500, 1380, 1310, 750 cm^{-1}. Nmr (CD_3OD) δ 1.60 (4H, m), 2.32 (2H, t, J = 7.0), 3.28 (2H, m), 3.90 (3H, s), 7.24 (1H, d, J = 1.8), 7.78 (1H, d, J = 1.8), 8.31 (0.5H, bt). FAB-MS 270 (MH^+, 31%). Anal. calcd. for $C_{11}H_{15}N_3O_5$: C, 48.58; H, 5.57; N, 15.45. Found: C, 48.56; H, 5.44; N, 15.50.

6-(1-Methyl-4-nitropyrrole-2-carboxamido)caproic acid (61) The procedure is similar to that of preparing 59. Yield 84%. Mp 154-155 °C. Ir (KBr) 3340, 1750, 1630, 1550, 1530, 1310, 1200, 750 cm^{-1}. Nmr (CD_3OD) δ 1.40 (2H, m), 1.60 (4H, m), 2.30 (2H, t, J = 7.0), 3.29 (2H, t, J = 7.0), 3.93 (3H, s), 7.27 (1H, d, J = 1.8), 7.82 (1H, d, J = 1.8). FAB-MS 284 (MH^+, 15%). Anal. calcd. for $C_{12}H_{17}N_3O_5$: C, 50.87; H, 6.06; N, 14.84. Found: C, 50.68; H, 6.23; N, 14.56.

7-(1-Methyl-4-nitropyrrole-2-carboxamido)heptanoic acid (62) The procedure is similar to that of preparing 59. Yield 89%. Mp 130-131 °C. Ir (KBr) 3340, 2940, 1740, 1630, 1550, 1520, 1490, 1390, 1310, 1210, 750, 600 cm^{-1}. Nmr (DMSO-d_6) δ 1.28 (4H, m), 1.49 (4H, m), 2.21 (2H, t, J = 7.0), 3.18 (2H, m), 3.92 (3H,s), 7.43 (1H, d, J = 1.8), 8.13 (1H, d, J = 1.8), 8.38 (1H, t, J = 5.8). FAB-MS 298 (MH^+, 12.6%). Anal. calcd. for $C_{13}H_{19}N_3O_5$: C, 52.52; H, 6.44; N, 14.13. Found: C, 52.72; H, 6.51; N, 14.07.

8-(1-Methyl-4-nitropyrrole-2-carboxamido)caprylic acid (63) The procedure is similar to that above. Yield 84%. Mp 140-141 °C. Ir (KBr) 3340, 3120, 3110, 2950, 1750, 1630, 1550, 1530, 1510, 1320, 1300, 810, 750 cm^{-1}. Nmr (CD_3OD) δ 1.37 (6H, m), 1.60 (4H, m), 2.26 (2H, t, J = 7.0), 3.30 (2H, m), 3.94 (3H, s), 7.28 (1H, d, J=1.8), 7.82 (1H, d, J=1.8). FAB-MS 312 (MH^+, 27%). Anal. calcd. for $C_{14}H_{21}N_3O_5$: C, 53.46; H, 6.74; N, 13.36. Found: C, 53.18; H, 6.75; N, 13.27.

4-[1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]butyric acid (69). A suspension of 59 (0.763 g, 3.00 mmol) and 10%

Pd/C (0.326 g) in MeOH was stirred in H_2 (1 atm) for 4 h. The catalyst was filtered and washed with MeOH and the combined filtrate was concentrated to a small volume. DMF (2 ml) was added and evaporated in high vacuum. To the amine residue dissolved in DMF (5 ml) and cooled to -20 °C, triethylamine(0.42 ml, 3 mmol) was added, followed by (O_2N)-Pyr-CCl_3 (0.816 g, 3 mmol) in THF (2 ml). The mixture was stirred at r.t. for 18 h, and the solvent was removed. Ethanol was added, and the resultant yellow crystals collected (0.625 g, 55% yield). Recrystallization from ethanol gave analytically pure crystals. Mp 251-252 °C. Ir (KBr) 3390, 3232, 3130, 1730, 1660, 1640, 1600, 1510, 1490, 1420, 1160 cm^{-1}. Nmr (DMSO-d_6) δ 1.70 (2H, dd, J = 7.0), 2.16 (2H, t, J = 7.0), 3.16 (2H, m), 3.80 (3H, s), 3.94 (3H, s), 6.85 (1H, d, J = 1.8), 7.20 (1H, d, J = 1.8), 7.57 (1H, d, J = 1.8), 8.17 (1H, d, J = 1.8), 8.25 (1H, bt), 10.28 (1H, s). FAB-MS 377 (MH^+, 4.34%). Anal. calcd. for $C_{16}H_{19}N_5O_6$: C, 50.16; H, 4.99; N, 17.78. Found: C, 50.05; H, 4.96; N, 17.81.

5-[1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]valeric acid (70). The procedure is similar to that above. Yield 40%. Mp 227-228 °C. Ir (KBr) 3410, 3140, 1740, 1640, 1600, 1520, 1310, 1170 cm^{-1}. Nmr (CD_3OD) δ 1.65 (4H, m), 2.35 (2H, t, J = 7.0), 3.31(2H, m), 3.86 (3H, s), 3.99 (3H, s), 6.79 (1H, d, J = 1.8), 7.20 (1H, d, J = 1.8), 7.40 (1H, d, J = 1.8), 7.86 (1H, d, J = 1.8). FAB-MS 392 (MH^+, 5%). Anal. calcd. for $C_{17}H_{21}N_5O_6$: C, 51.51; H, 5.18; N, 17.01. Found: C, 51.18; H, 5.30; N, 17.23.

6-[1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]caproic acid (71). The procedure is similar to that above. Yield 61%. Mp 245-246°C. Ir (KBr) 3400, 3120, 2930, 1730, 1640, 1600, 1510, 1410, 1400, 1310, 1250, 1150, 750 cm^{-1}. Nmr (DMSO-d_6) δ 1.27 (2H, m), 1.48 (4H, m), 2.20 (2H, t, J = 7.2), 3.13 (2H, m), 3.79 (3H, s), 3.94 (3H, s), 6.83 (1H, d, J = 1.8), 7.18 (1H, d, J = 1.8), 7.57 (1H, d, J = 1.8), 8.03 (1H, t, J = 6.0), 8.17 (1H, d, J = 1.8), 10.20 (1H, s).

FAB-MS 406 (MH^+, 1%). Anal. calcd. for $C_{18}H_{23}N_5O_6$: C, 52.53; H, 5.64; N, 17.02. Found: C, 52.84; H, 5.74; N, 16.68.

7-[1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]heptanoic acid (72). The procedure similar to that above. Yield 50%. Mp 209-210 °C. Ir (KBr) 3400, 3130, 2940, 1710, 1670, 1580, 1530, 1500, 1420, 1310, 1180, 1130, 810 cm^{-1}. Nmr (DMSO-d_6) δ 1.27 (4H, bs), 1.47 (4H, m), 2.18 (2H, t, J = 7.2), 3.14 (2H, m), 3.79 (3H, s), 3.94 (3H, s), 6.83 (1H, d, J = 1.8), 7.18 (1H, d, J = 1.8), 7.57 (1H, d, J = 1.8), 8.03 (1H, t, J = 6.0), 8.17 (1H, d, J = 1.8), 10.21 (1H, s). FAB-MS 420 (MH^+, 0.9%). Anal. calcd. for $C_{19}H_{25}N_5O_6$: C, 53.86; H, 5.95; N, 16.53. Found: C, 54.07; H, 5.91; N, 16.37.

8-[1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]caprylic acid (73). The procedure is similar to that above. Yield 57%. Mp 218-220 °C. Ir (KBr) 3400, 3250, 3140, 2940, 1730, 1640, 1590, 1510, 1490, 1300, 1210, 1150, 1120, 820, 750 cm^{-1}. Nmr (DMSO-d_6) δ 1.27 (6H, bs), 1.48 (4H, m), 2.18 (2H, t, J = 7.2), 3.14 (2H, m), 3.79 (3H, s), 3.94 (3H, s), 6.82 (1H, d, J = 1.8), 7.19 (1H, d, J = 1.8), 7.57 (1H, d, J = 1.8), 8.03 (1H, t, J = 6.0), 8.17 (1H, d, J = 1.8), 10.21(1H, s). FAB-MS 434 (MH^+, 0.7%). Anal. calcd. for $C_{20}H_{27}N_5O_6$: C, 55.42; H, 6.28; N, 16.16. Found: C, 55.49; H, 6.21; N, 15.87.

1-Methyl-4-{1-methyl-4-[1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamidoacetamido]pyrrole-2-carboxamido}-pyrrole-2-carboxamidopropionamidine hydrochloride (75). A solution of 40 (1.01 g, 2.53 mmol) and 10% Pd/C (0.268 g, 2.53 mmol) in DMF/MeOH (10ml/10ml) was stirred in *Paar* Shaker (H_2, 70 psi) at r.t for 4 h. The catalyst was filtered and washed thoroughly with MeOH, the solvent was evaporated in vacuo then high vacuum until most of the DMF was removed. The amine residue dissolved in DMF (3 ml) was cooled to -10 °C, then 47 (0.889 g, 2.53 mmol) was added, followed by DMAP (0.309 g) and DCC

(0.522 g). The solution was brought up to r.t. and the stirring was continued for 18 h. Solvent was removed, and the residue was dissolved in MeOH and precipitated with ether to give crude product (1.20 g). Purification with reversed phase HPLC (C_4) gave pure solid (0.299 g, 17% yield). Mp 185 °C (dec.). Ir (KBr) 3370, 3105, 1640, 1580, 1535, 1440, 1310, 1210 cm^{-1}. Nmr (DMSO-d_6) δ 2.53 (2H, t, J = 7.0), 3.45 (2H, m), 3.79 (3H, s), 3.81 (3H, s), 3.82 (3H, s), 3.88 (2H, d, J = 5.8), 3.93 (1H, s), 6.88 (1H, d, J=1.9), 6.90 (1H, d, J = 1.9), 6.94 (1H, d, J = 1.9), 7.17 (1H, d, J = 1.9), 7.20 (1H, d, J = 1.9), 7.27 (1H, d, J = 1.9), 7.58 (1H, d, J = 1.9), 8.18 (1H, d, J = 1.9), 8.27 (1H, t, J = 5.8), 8.40 (1H, t, J = 5.8), 9.89 (1H, s), 9.94 (1H, s), 10.32 (1H, s). FAB-MS: calcd. mass for $C_{29}H_{35}N_{12}O_7$ (M-Cl) 663.2751. Found *via* exact FAB-MS: 663.2761 (5.6%).

1-Methyl-4-{1-methyl-4-{3-[1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]propionamido}pyrrole-2-carboxamido}pyrrole-2-carboxamidopropionamidine hydrochloride (76) Procedure is similar to that above. Yield 13%. No distinct m.p. Ir (KBr) 3300, 3120, 1640, 1535, 1405, 1310 cm^{-1}. Nmr (DMSO-d_6) δ 2.53 (2H, t, J = 7.0), 3.38 (2H, m), 3.44 (2H, m), 3.79 (3H, s), 3.80 (6H, s), 3.93 (3H, s), 6.82 (1H, d, J = 1.9), 6.85 (1H, d, J = 1.9), 6.88 (1H, d, J = 1.9), 7.18 (1H, d, J = 1.9), 7.22 (1H, d, J = 1.9), 7.57 (1H, d, J = 1.9), 8.14 (1H, d, J =1.9), 8.17 (1H, d, J = 1.9), 8.25 (1H, t, J = 5.9), 9.90 (1H, s), 9.94 (1H, s), 10.26 (1H, s). FAB-MS: calcd. mass for $C_{30}H_{37}N_{12}O_7$ (M-Cl) 677.2908. Found *via* exact FAB-MS: 677.2929 (1.9%).

1-Methyl-4-{1-methyl-4-{1-methyl-4-[1-methyl-4-(formylamino) pyrrole-2-carboxamido]pyrrole-2-carboxamidoacetamido}pyrrole-2-carboxamido}pyrrole-2-carboxamidopropionamidine hydrochloride (77) A suspension of 10% Pd/C (0.073 g) and crude 75 (0.120 g) in 15 ml of MeOH was shaken vigorously in hydrogen bomb (600 psi) for 2 h. The catalyst was removed by filtration,

then washed with MeOH, and the combined filtrate and washings was evaporated in *rotavapor* then in high vacuum. The amine residue was dissolved in DMF (2 ml). A solution of 98% formic acid (0.033 ml, 0.885 mmol) was added to CDI (0.139 g, 0.855 mmol) suspended in THF (2 ml). After 0.5 h's stirring at r.t., 0.6 ml of this prepared solution was added to the amine residue cooled to -40 °C. The mixture was stirred at -40 °C for 45 min. then brought up to r.t. Solvent was removed and the residue was dissolved in small amount of MeOH, then precipitated by ether. Purification by reversed phase HPLC (C_4) gave analytically pure solid (11 mg, 37% yield). No distinct m.p. Ir (KBr) 1640, 1580, 1530, 1430, 14[illegible] cm^{-1}. Nmr (DMSO-d_6) δ 2.54 (2H, t, J = 7.0), 3.46 (2H, m), 3.80 (3H, s), 3.[illegible]), 3.83 (3H, s), 3.84 (1H, s), 3.90 (2H, d, J = 6.0), 6.88 (1H, d, J = 1.9), 6.9[illegible]t, d, J = 1.9), 6.94 (1H, d, J = 1.9), 7.17 (1H, d, J = 1.9), 7.19 (1H, d, J = 1.9), 7.23 (1H, d, J = 1.9), 8.12 (1H, d, J = 1.9), 8.27 (1H, d, J = 1.9), 8.32 (1H, t, J = 6.0), 9.90 (1H, s), 9.92 (1H, s), 9.94 (1H, s), 10.32 (1H, s). FAB-MS: calcd. mass for $C_{30}H_{37}N_{12}O_6$ (M-Cl) 661.2959. Found *via* exact FAB-MS: 661.2989 (0.56%).

1-Methyl-4-{1-methyl-4-{3-{1-methyl-4-[1-methyl-4-(formylamino)-pyrrole-2-carboxamido]pyrrole-2-carboxamido}propionamido}pyrrole-2-carbox-amido}pyrrole-2-carboxamidopropionamidine hydrochloride (78). Procedure is similar to that above. Yield 43%. No distinct m.p. Ir (KBr) 1641, 1580, 1528, 1450, 1435, 1261 cm^{-1}. Nmr (DMSO-d_6) δ 2.51(2H, t, J = 7.0), 2.53 (2H, t, J = 7.0), 3.38 (2H, m), 3.44 (2H, m), 3.78 (3H, s), 3.82 (6H, s), 6.82 (1H, d, J = 1.9), 6.86 (1H, d, J = 1.9), 6.88 (2H, m), 7.17 (4H, m), , 8.07 (1H, t, J = 6.0), 8.10 (1H, s), 8.26 (1H, t, J = 6.0), 9.88 (1H, s), 9.89 (1H, s), 9.93 (1H, s), 10.08 (1H, s). FAB-MS: calcd. mass for $C_{31}H_{39}N_{12}O_6$ (M-Cl) 675.3115. Found *via* exact FAB-MS: 675.3164 (0.14%).

4-{1-Methyl-4-[1-methyl-4-(formylamino)pyrrole-2-carboxamido] pyrrole-2-carboxamido}butyric acid (81). A suspension of 69 (0.407 g, 1.08 mmol) and 10% Pd/C (0.114 g) in DMF/MeOH (10 ml/10 ml) was stirred in H_2(1 atm) for 2 h. Catalyst was removed by filtration and washed with MeOH, then the combined filtrates and washings was concentrated to a small volume. To a suspension of CDI (0.787 g, 4.85 mmol) in 3 ml THF, HCOOH (0.185 ml) was added, and the solution was stirred at r.t. for o.5 h then added dropwise to the amine residue cooled to -40 °C. Stirring was continued at -40 °C for 45 min. then at r.t. for 18 h. DMF was removed, MeOH was added, and the product was precipitated with ether. Purification with reversed phase HPLC yielded pure 81. No distinct m.p. Ir (KBr) 3400, 2960, 1300, 1260, 1100, 1020, 800 cm^{-1}. Nmr (DMSO-d_6) δ 1.70 (2H, m), 2.22(2H, t, J = 7.0), 3.18 (2H, m), 3.83 (3H, s), 3.78 (3H, s), 6.87 (2H, bd), 6.90 (1H, bd), 7.18 (1H, bd), 7.20 (1H, bd), 8.10 (1H, t, J = 5.5), 8.13 (1H, s), 9.88 (1H, s), 10.07 (1H, s). Calcd. mass for $C_{17}H_{22}N_5O_5(MH^+)$: 376.1621. Found *via* exact mass FAB: 376.1634 (4.3%).

5-{1-Methyl-4-[1-methyl-4-(formylamino)pyrrole-2-carboxamido] pyrrole-2-carboxamido}valeric acid (82). Procedure as above. Yield 70% Mp: 115 °C (dec.) Ir (KBr) 3380, 3300, 2920, 1670, 1530, 1440, 1400, 1260, 1210 cm^{-1}. Nmr (DMSO-d_6) δ 1.50 (4H, bs), 2.22 (2H, t, J = 7.0), 3.16 (2H, m), 3.78 (3H, s), 3.82 (3H, s), 6.34 (1H, d, J=1.8), 6.90 (1H, d, J = 1.8), 7.17 (1H, d, J = 1.8), 7.20 (1H, d, J = 1.8), 8.01(1H, t, J = 5.7), 8.12 (1H, d,J = 1.4), 9.89 (1H, s), 10.07 (1H, s). Calcd. mass for $C_{18}H_{24}N_5O_5$ (MH^+): 390.1777. Found *via* exact mass FAB: 390.1773 (6.8%).

6-{1-Methyl-4-[1-methyl-4-(formylamino)pyrrole-2-carboxamido] pyrrole-2-carboxamido}caproic acid (83). Procedure as above. Yield 71%. Mp: 135°C (dec.). Ir(KBr) 3290, 2930, 1640, 1580, 1540, 1440, 1400, 1270, 1130, 770 cm^{-1}. Nmr (DMSO-d_6) δ 1.27 (2H, m), 1.48 (4H, m), 2.17 (2H, t, J = 7.5), 3.13 (2H, m), 3.76(3H, s), 3.81(3H, s), 6.83(1H, d, J = 1.8), 6.89 (1H, d, J = 1.8), 7.16(1H, d, J =

1.8), 7.18 (1H, d, J = 1.8), 7.98 (1H, t, J = 5.8), 8.12 (1H, d, J = 1.8), 9.86 (1H, s), 10.07 (1H, s). Calcd. mass for $C_{19}H_{26}N_5O_5$ (MH^+): 404.1934. Found *via* exact mass FAB: 404.1939 (14.1%).

7-{1-Methyl-4-[1-methyl-4-(formylamino)pyrrole-2-carboxamido] pyrrole-2-carboxamido}heptanoic acid (84). Procedure as above. Yield 76%. Mp:172-174°C (dec.). Ir (KBr) 3370, 2950, 1690, 1630, 1540, 1440, 1400, 1290, 1200, 1120, 1090, 810, 760 cm^{-1}. Nmr (DMSO-d_6) δ 1.27 (4H, m), 1.47(4H, m), 2.18(2H, t, J = 7.2), 3.13 (2H, m), 3.77 (3H, s), 3.81(3H, s), 6.82(1H, d, J = 1.8), 6.88 (1H, d, J = 1.8), 7.16 (1H, d, J = 1.8), 7.18 (1H, d, J = 1.8), 7.98 (1H, t, J = 5.8), 8.11 (1H, d, J = 1.8), 9.87 (1H, s), 10.09 (1H, s). Calcd. mass for $C_{20}H_{28}N_5O_5$ (MH^+):418.2090. Found *via* exact mass FAB: 418.2074 (33%).

8-{1-Methyl-4-[1-methyl-4-(formylamino)pyrrole-2-carboxamido] pyrrole-2-carboxamido}caprylic acid (85). Procedure as above. Yield 62%. Mp:179-181°C (dec.). Ir (KBr) 3370, 2930, 2840, 1700, 1670, 1620, 1540, 1440, 1260, 1210, 1130, 1090, 860, 820, 760 cm^{-1}. Nmr (DMSO-d_6) δ 1.26 (6H, m), 1.47 (4H, m), 2.14 (2H, t, J = 7.2), 3.13 (2H, m), 3.77 (3H, s), 3.81 (3H, s), 6.82 (1H, d, J = 1.8), 6.88 (1H, d, J = 1.8), 7.16(1H, d, J = 1.8), 7.18 (1H, d, J = 1.8), 7.98 (1H, t, J = 5.8), 8.11(1H, d, J = 1.8), 9.87 (1H, s), 10.09 (1H, s). Calcd. mass for $C_{21}H_{30}N_5O_5$ (MH^+): 432.2247. Found *via* exact mass FAB: 432.2222 (33%).

1-Methyl-4-{1-methyl-4-{4-{1-methyl-4-[1-methyl-4-(formylamino) pyrrole-2-carboxamido]pyrrole-2-carboxamido}butyramido}pyrrole-2-carboxamido}pyrrole-2-carboxamidopropionamidine hydrochloride (86). A suspension of amidine 40 (0.139 g, 0.35 mmol) and 10% Pd/C (0.055 g) in MeOH/DMF (10 ml/10 ml) was stirred in H_2 (60 psi) on *Paar* Shaker apparatus for 4 h. The catalyst was filtered and washed with MeOH, then the filtrate was concentrated to a small volume. To the amine residue cooled to 0 °C, acid 81 (0.131 g, 0.35 mmol), DMAP (0.045 g, 0.36

mmol), and DCC (0.073 g, 0.35 mmol) was added. The solution was brought up to r.t. and stirred under argon for 18 h. Solvent was removed, MeOH was added, followed by ether, and the resultant solid collected (0.165 g). Purification with reversed- phase HPLC yielded pure 86 (0.041 g, 16% yield). No distinct m.p. Ir (KBr) 3280, 2930, 1640, 1580, 1530, 1440, 1400, 1270, 1210, 770 cm^{-1}. Nmr (DMSO-d_6) δ 1.78 (1H, quintet, J = 7.2), 2.27 (2H, t, J = 7.5), 2.54 (2H, t, J = 6.6), 3.20 (2H, m), 3.46 (2H, m), 3.80 (3H, s), 3.81 (6H, s), 3.83 (3H, s), 6.85 (1H, d, J = 1.8), 6.89 (3H, m), 7.17 (2H, distd. t), 7.19 (2H, distd. t), 8.04 (1H, t, J = 5.5), 8.12 (1H, s), 8.25 (1H, t, J = 5.5), 9.84 (1H, s), 9.87 (2H, s), 10.07 (1H, s). FAB-MS: calcd. mass for $C_{32}H_{41}N_{12}O_6$ (M-Cl) 689.3272. Found *via* exact FAB-MS: 689.3239 (1.8%).

1-Methyl-4-{1-methyl-4-{5-{1-methyl-4-[1-methyl-4-(formylamino) pyrrole-2-carboxamido]pyrrole-2-carboxamido}valeramido}pyrrole-2-carboxamido}pyrrole-2-carboxamidopropionamidine hydrochloride (87)
The procedure as above. Yield 12%. No distinct m.p. Ir (KBr) 3320, 1640, 1580, 1530, 1440, 1400, 1270, 1200, 1100, 770 cm^{-1}. Nmr (DMSO-d_6) δ 1.49 (2H, m), 1.59 (2H, m), 2.25 (2H, t, J = 7.0), 2.53 (2H, t, J = 6.8), 3.17 (2H, m), 3.47 (2H, m), 3.78 (3H, s), 3.79 (3H, s), 3.80 (3H, s), 3.82 (3H, s), 6.84 (1H, d, J = 1.8), 6.88 (2H, distd. d), 7.15 (2H, d, J = 1.8), 7.18 (2H, distd. t), 8.01 (1H, t, J = 6.0), 8.11(1H, s), 8.23(1H, t, J = 6.0), 9.77 (1H, s), 9.85 (1H, s), 10.07 (1H, s). FAB-MS: calcd. mass for $C_{33}H_{43}N_{12}O_6$(M-Cl) 703.3428. Found *via* exact FAB-MS: 703.3447 (0.9%).

1-Methyl-4-{1-methyl-4-{6-{1-methyl-4-[1-methyl-4-(formylamino) pyrrole-2-carboxamido]pyrrole-2-carboxamido}caproamido}pyrrole-2-carboxamido}pyrrole-2-carboxamidopropionamidine hydrochloride (88).
The procedure as above. Yield 11%. No distinct m.p. Ir (KBr) 3400, 1640, 1580, 1530, 1440, 1400, 1260, 1200, 1140, 1100, 770 cm^{-1}. Nmr (DMSO-d_6) δ 1.31(2H, m), 1.50 (2H, m), 1.59 (2H, m), 2.23 (2H, t, J = 7.0), 2.54 (2H, t, J = 6.8), 3.16 (2H, m),

3.47 (2H, m), 3.78 (3H, s), 3.80 (3H, s), 3.81 (3H, s), 3.83 (3H, s), 6.85 (2H, distd. t), 6.89 (2H, distd. d), 7.15 (2H, distd. t), 7.18 (2H, distd. t), 8.00 (1H, t, J = 6.0), 8.12 (1H, s), 8.27 (1H, t, J = 6.0), 9.79 (1H, s), 9.88 (2H, s), 10.07 (1H, s). FAB-MS: calcd. mass for $C_{34}H_{45}N_{12}O_6$(M-Cl) 717.3585. Found *via* exact FAB-MS: 717.3611 (1.4%).

1-Methyl-4-{1-methyl-7-{4-{1-methyl-4-[1-methyl-4-(formylamino) pyrrole-2-carboxamido]pyrrole-2-carboxamido}heptanoamido}pyrrole-2-carboxamido}pyrrole-2-carboxamidopropionamidine hydrochloride (89). The procedure as above. Yield 16%. No distinct m.p. Ir (KBr) 3290, 2940, 1640, 1580, 1530, 1440, 1400, 1260, 1200, 1140, 1100, 770 cm^{-1}. Nmr (DMSO-d_6) δ 1.31(4H, bs), 1.47 (2H, bt), 1.57 (2H, bt), 2.22 (2H, t, J = 7.0), 2.58 (2H, bt), 3.15 (2H, m), 3.49 (2H, m), 3.77 (3H, s), 3.79 (3H, s), 3.80 (3H, s), 3.81(3H, s), 6.83 (1H, bd), 6.85 (1H, bs), 6.88 (1H, bd), 6.92 (1H, bs), 7.12 (1H, bs), 7.14 (1H, bd), 7.16 (1H, bd), 7.94 (1H, t, J = 6.0), 8.12 (1H, s), 8.15 (1H, bs), 8.40 (1.5H, bs), 8.87 (1.3H, bs), 9.72 (1H, s), 9.82 (2H, s), 10.00 (1H, s). FAB-MS: calcd. mass for $C_{35}H_{47}N_{12}O_6$ (M-Cl) 731.3741. Found *via* exact FAB-MS: 731.3773 (4.7%).

1-Methyl-4-{1-methyl-4-{8-{1-methyl-4-[1-methyl-4-(formylamino) pyrrole-2-carboxamido]pyrrole-2-carboxamido}caprylamido}pyrrole-2-carboxamido}pyrrole-2-carboxamidopropionamidine hydrochloride (90). The procedure as above. Yield 12%. No distinct m.p. Ir (KBr) 3280, 2930, 1640, 1580, 1530, 1440, 1400, 1260, 1200, 1140, 1100, 770 cm^{-1}. Nmr (DMSO-d_6) δ 1.31 (6H, bs), 1.48 (2H, m), 1.57 (2H, m), 2.23 (2H, t, J = 7.2), 2.55 (2H, t, J = 6.6), 3.15 (2H, m), 3.47 (2H, m), 3.78 (3H, s), 3.80 (3H, s), 3.81 (3H, s), 3.83 (3H, s), 6.84 (1H, d, J = 1.8), 6.86 (1H, d, J = 1.8), 6.90 (1H, d, J = 1.8), 7.16 (2H, distd. t), 7.19 (2H, distd. t), 7.98 (1H, t, J = 5.5), 8.13 (1H, s), 8.26 (1H, t, J = 5.8), 9.79 (1H, s),

9.88 (2H, s), 10.10 (1H, s). FAB-MS: calcd. mass for $C_{36}H_{49}N_{12}O_6$ (M-Cl) 745.3898. Found *via* exact FAB-MS: 745.3826 (1.8%).

Chapter 3

Biological Studies on Head-to-tail Linked Bis-lexitropsins

I. Footprinting

I. A Biochemicals

Restriction enzymes ECoRI and Hind III, sonicated calf thymus (phenol extracted) and pBR322 DNAs, poly(dA-dT), poly(dG-dC) were purchased from Pharmacia P. L. Biochemicals. All were used without further purification. The T_4 polynucleotide kinase, Klenow fragment (large fragment of DNA polymerase I) and urea were purchased from Bethesda Research Labs. Calf intestine alkaline phosphatase(CAP) and dithiothreitol (DDT) were obtained from Calbiochem. γ-^{32}P-ATP and α-^{32}P-dATP were purchased from New England Nuclear. Methidium-propyl-EDTA(MPE) was a gift from Professor P. B. Dervan(Cal. Tech.). All other reagents were of analytical grade.

I. B Footprinting procedure*

The ECoRI digested pBR322 DNA was labelled either at 3'-end using γ-^{32}P-dATP and Klenow fragment of DNA polymerase or at 5'-end using α-^{32}P-ATP and T4 polynucleotide kinase. The resultant 3'- or 5'-labelled fragments were digested with Hind III. The footprinting reaction were performed on either 3'- or 5'-end labelled pBR322 DNA fragments in the presence of sonicated calf thymus DNA and the new set of ligands in buffer consisting of 10 mM Tris, 20 mM NaCl (pH 7.4). Control sets were performed exactly as above with the exception that ligand was omitted from the reaction mixture. The ligand-DNA reaction contents were incubated at 37 °C for 30 minutes, followed by successive addition of MPE•Fe(II)(freshly prepared) and dithiotheitol (DDT). After 15 minutes, the reactions were stopped by instant freezing at -70 °C. The final reaction

contents containing 100 mM DNA, 10 mM Tris, 20 mM NaCl, 10 mM MPE•Fe(II), 2.5 mM DTT and 8 and 32 mM of ligands. The solutions were lyophilized, and resuspended in formamide loading buffer and heated at 90 °C prior to gel electrophoresis. The samples were loaded on 0.4 mm thick , 55 cm long 6% polyacrylamide and 7 M urea denaturing gel at 2000 V and 55 °C. At the end of electrophoresis, the gel was dried on a Bio-Rad model 483 slab dryer. The dried gel was autoradiographed at -70 °C using Kodak X-Omat AR film without intensifying screen. The resultant autoradiograms were scanned on LKB ultrascan XL laser densitometer interfaced with a Zenith-Z-100 personal computer. Background absorbance of the film(<0.35 absorbance) was substracted from all the band intensities. All band intensities within a lane were normalized to a band which is unaffected by ligand-DNA interaction to compensate for possible variation in extent of reaction. The measure of protection from cleavage for each base was determined by

$$\% \text{ Protection} = [1-(A_{\text{ligand+DNA}}/A_{\text{DNA}}]*100$$

where $A_{\text{ligand+DNA}}$ is the absorbance of a band obtained from cleavage in the presence of added ligand and A_{DNA} is the absorbance of the same band in the absence of the ligand. The autoradiograms and histograms of footprinting results are shown in Figures 12 to 17.

*Footprinting was performed by Dr. Rajan Gupta at Department of Chemistry, University of Alberta.

II Binding Constants for Ligand-DNA Interaction

Relative binding constants of ligands to poly(dA-dT), calf thymus DNA and poly(dG-dC) were estimated by measuring the decrease in fluorescence of an ethinium-DNA complex as a result of competitive displacement (Table 5).[75] The concentration that produced 50% inhibition of fluorescence was taken to be inversely proportional to the binding constant. The fluorescence displacement studies were made on a Turner 430

Removed due to poor print quality

Figure 12. A portion of the footprinting autoradiogram from MPE•Fe(II) cleavage of 5'-^{32}P end labelled EcoRI/Hind III restriction fragment of pBR322. The lane labelled DNA contains the intact DNA fragment. Control lane is MPE•Fe(II) cleavage in the absence of ligands. The other lanes contain **77**, **78**, **86**, or distamycin at r = 0.08 and r = 0.32 as indicated as well as G reaction.

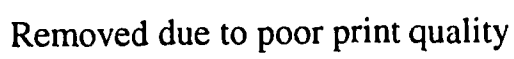


Figure 13. A portion of the footprinting autoradiogram from MPE•Fe(II) cleavage of 3'-^{32}P end labelled EcoRI/Hind III restriction fragment of pBR322. Lanes contain ligands or controls as indicated at r = 0.08 or r = 0.32.

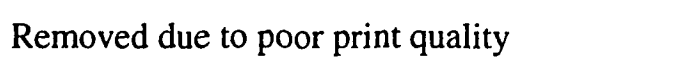


Figure 14. A portion of the footprinting autoradiogram from MPE•Fe(II) cleavage of 5'-^{32}P end labelled EcoRI/Hind III restriction fragment of pBR322. Control lane is MPE•Fe(II) cleavage in the absence of ligands. The other lanes contain **87**, **88**, **89**, **90**, or distamycin at r = 0.08 and r = 0.32 as indicated as well as G reaction.

Removed due to poor print quality

Figure 15. A portion of the footprinting autoradiogram from MPE•Fe(II) cleavage of 3'-^{32}P end labelled EcoRI/Hind III restriction fragment of pBR322. Lanes contain ligands at r = 0.08 or r = 0.32 or controls as needed.

Figure 16. Footprinting of A (77), B (78), C (86), D (87), E (88), F (89), G (60), and H (distamycin). Histogram height is proportional to the protection from the cleavage at each base pair relative to unprotective DNA (in the absence of ligands). Ligand to DNA base pair ratio is 0.08. Upper and lower footprints are from 5'- and 3'-end labelled DNA respectively.

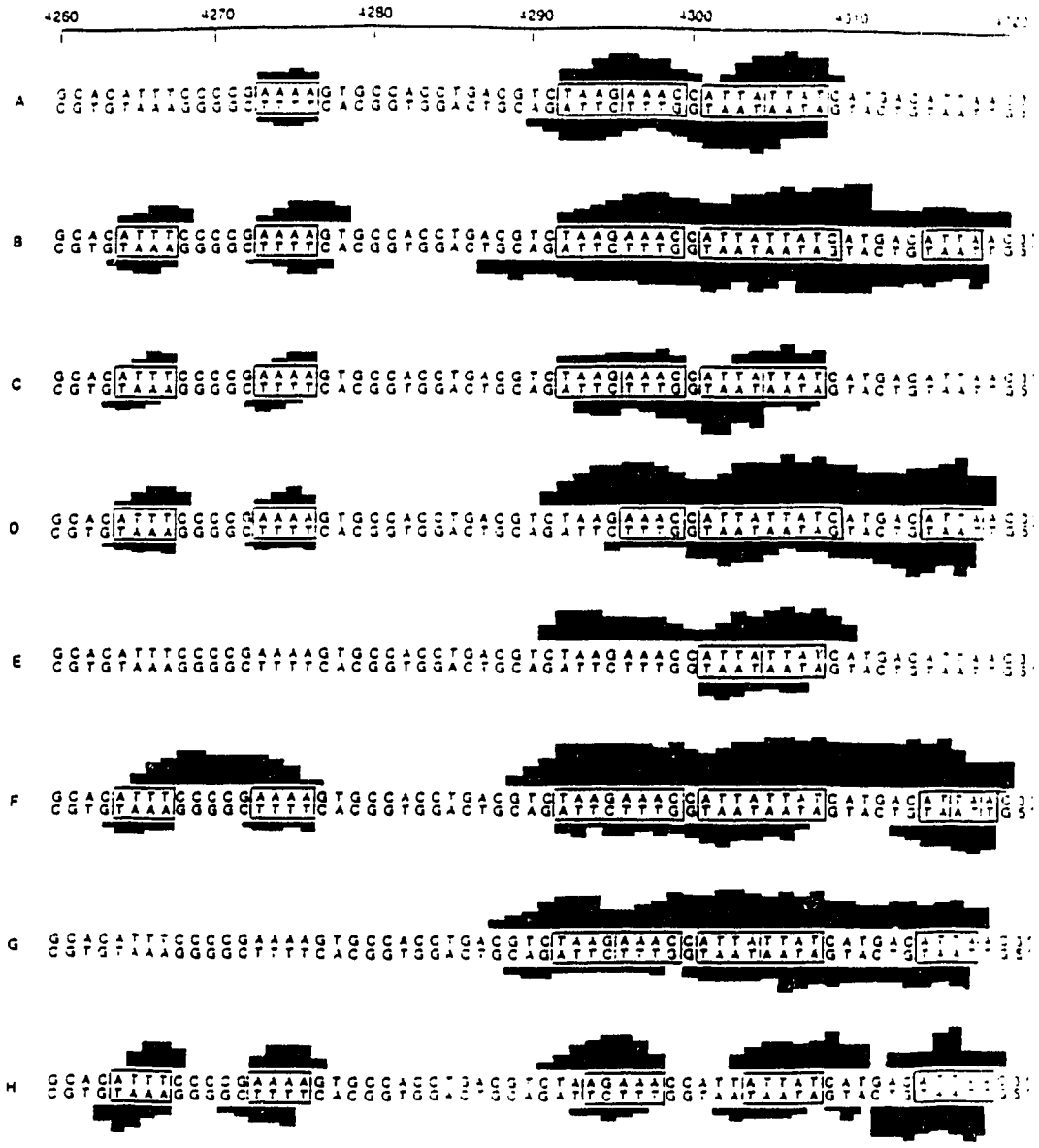


Figure 17. Footprinting of A (77), B (78), C (86), D (87), E (88), F (89), G (60), and H (distamycin). Histogram height is proportional to the protection from the cleavage at each base pair relative to unprotective DNA (in the absence of ligands). Ligand to DNA base pair ratio is 0.32. Upper and lower footprints are from 5'- and 3'-end labelled DNA respectively.

spectrofluorometer with excitation and emission wavelength of 525 and 600 nm respectively. All the experiments were performed in 10 mM Tris, 20 mM NaCl buffer (pH 7.4), and at room temperature of 21 ±1 °C.

Table 5 Relative binding constants of distamycin and new series of compounds as determined by ethidium displacement assay.[a]

	DNA binding constant, M^{-1}		
Compounds	poly(dA-dT)	ct DNA	poly(dG-dC)
Distamycin	0.55×10^8	1.11×10^6	6.52×10^5
47	0.28×10^8	0.71×10^6	2.32×10^5
48	7.69×10^8	0.66×10^6	0.90×10^5
56	0.08×10^8	0.32×10^6	2.94×10^5
57	8.33×10^8	0.80×10^6	4.43×10^5
58	0.29×10^8	1.25×10^6	6.25×10^5
59	16.66×10^8	2.22×10^6	4.54×10^5
60	0.62×10^8	1.11×10^6	7.10×10^5

[a] Based on binding constant of ethidium bromide of 10^7 M^{-1} under similar conditions. Binding constant values represent the average of two experiments.

III. Cytotoxicity Assay

III. A Materials

In Vitro KB cell cytotoxicity assay[76] was performed at SynPhar Laboratories Inc., Edmonton. The materials used are: KB cells (ATCC CCL 17); minimum Essential Medium, Eagles (modified with Earles salt) supplemented with 10% calf serum, 100 $IU.ml^{-1}$ penicillin G, 100 $mg.ml^{-1}$ streptomycin; compounds dissolved in DMSO to 20

$mg.l^{-1}$ and further diluted in the CS-MEM; crystal violet; 25% glutaraldehyde; deionized water.

III. B Method

The method employed was a modification of the crystal violet assay. KB cells were cultivated in Eagles minimum essential medium supplemented with 10% calf serum and incubated at 37 °C in a humidified 5% CO_2 atmosphere to prepare a cell stock. Cells were counted using a Neubauer hemocytometer and seeded in 96 well plates at 100 ml of 3×10^3 cells per ml and cultured for one day. Test compounds were diluted and 100 ml of the solution was added in triplicate wells to give final concentrations of 10, 5, 1, 0.5 and 0.1 μml^{-1}. Control wells were identical except that the test compound was absent. These were cultured for 3 days. Then the cells were fixed with addition of 20 ml of 25% glutaraldehyde for 15 minutes, washed with water and dried. Then stained with 100 ml of 0.05% crystal violet for 15 minutes, washed with water and dried. The wells were eluted with 100 ml of 0.05 M NaH_2PO_4/ethanol (1:1 v/v) and read at OD_{540} on a multiscan spectrophotometer. ED_{50} values were calculated using the formula

$$\frac{[\%\text{ inhibition greater than } 50\% - 50\%]}{[\%\text{ inhibition greater than } 50\% - \%\text{ inhibition less than } 50\ \%]}$$

to give the interpolative value between two dilutions. The resulting data are shown in Table 6.

IV Antiviral Activity Assay

Since lexitropsins generally display wide-spectrum antiviral activity *in vitro*, the homologous series of head-to-tail linked bis-lexitropsins were submitted to antiviral tests

Table 6 In vitro KB cytotoxicity and antiviral activity against herpes simplex virus, moloney leukemia virus, influenza, reo virus for the new series of methylene linked lexitropsins

	KB	HSV		MLV		IFZ		RSV	
Compds.	TD_{50}	TD_{50}	MIC_{50}	TD_{50}	MIC_{50}	TD_{50}	MIC_{50}	TD_{50}	MIC_{50}
77	75.65	>100	>100	>100	>100	6.85	>1.0	>100	>100
78	9.66*	>100	>100	32.86	>10	5.97	>1.0	40	>10
86	>100	>100	>100	42.00	>10	>100	>100	>100	>100
87	52.52	>100	>100	>100	67.41#	5.52	>1.0	>100	>100
88	>100	>100	>100	>100	>100	74.75	>10	>100	>100
89	5.75*	>100	14.0@	61.43	>10	1.72	>1.0	55	>10
90	59.29	56.38	>10	65.38	>10	5.40	>1.0	>100	>100
ADR	0.0035								
ACK		>1.0	0.84						
AZT				>0.05	<0.001				
Amantadine						>50	0.175		
Ribavirin								26	4.5

* slightly toxic
@ slightly active against HSV
# slightly active against MLV

against herpes simplex virus, moloney leukemia virus, influenza, and reo virus and the results are summarized in Table 6.

V Results

V. A Binding of Linked Lexitropsins to Duplex DNA

The homologous series 77, 78, 86-90 bind to duplex DNA but not to single-stranded DNA. Relative binding constants of these agents and distamycin to calf thymus DNA, poly(dA-dT) and poly(dG-dC) were estimated and compared by measuring the decrease in fluorescence of an ethidium-DNA complex as a result of competitive displacement.[75] The drug concentration that produced 50% suppression of fluorescence was taken to be inversely proportional to the binding constant. Relative binding constants for this series of linked lexitropsins are shown in table 5. None of the drugs tested interfered with the fluorescence measurements at the concentration levels used, and all displaced ethidium from the DNA with relative binding constants to poly(dA-dT) ranging from $<0.1 \times 10^8$ to $16.6 \times 10^8\ M^{-1}$. The binding was influenced by the length of the linker with maxima at n = 2, 4, 6. Although the binding to poly(dA-dT) was stronger than to calf thymus DNA, in accord with the anticipated AT preference, a similar alternation of maxima and minima corresponding to even and odd values of n (except n = 4) is observed. However the binding pattern of this group of compounds with poly(dG-dC) is entirely different from those with poly(dA-dT) and calf thymus DNA with a maximum at n = 7.

V. B Sequence Selective DNA Binding

The linked bis-lexitropsins and distamycin have been investigated for their sequence selective binding to DNA on a EcoRI/Hind III cut restriction fragment of pBR322. The binding locations and binding site sizes on DNA have been determined by complementary strand MPE•Fe(II) footprinting.[77] The autoradiograms of the MPE•Fe(II) footprinting

assays at r = 0.08 and 0.32 on both 3'- and 5'-end labelled EcoRI/Hind III digested fragments of pBR322 are shown in Figures 12-15. The areas of decreased intensity compared with the control lane(without ligands) are due to the interaction of linked bis-lexitropsin that prevents MPE•Fe(II) cleavage on DNA. Densitometric analysis of the patterns of each lane permitted estimation of the location, approximate site size, and relative strength of binding of the linked bis-lexitropsin. Relative strength and binding site size of the protection at the binding sequences over the analyzed portion of the DNA fragment (bases 4260-4320) are shown in the form of histograms in Figures 16 and 17.

The autoradiograms and histograms revealed that the new compounds bind in different locations and have different binding site sizes from distamycin. The linked bis-lexitropsins show footprints mainly in the regions (all sequence from 5' to 3') at 4263-4269 [CATTCC], 4271-4278 [GAAAAGT] and to the analyzed portion on both the strands for compounds 77, 78, 86, at 4287-4310[ACGTCTAAGAAACCATTATTATCA], for compounds 87-90 at 4288-4317[CGTCTAAGAAACCATTATTATCATGAC TT]. At low ligand to DNA base pair ratio of r = 0.08, binding sites 4263-4269 and 4272-4278 were not observed with all the ligands except 87. At the higher ratio r =0.32, both these sites are clearly visible for 78, 86, 87, 89, and distamycin.

The footprints of distamycin at r = 0.08 and 0.32 are evident from 4293-4298 [AAGAAAA], 4302-4308 [TTATTAT] and 4311-4317 [TGACATT]. The binding locations and probable site sizes are determined on the basis of the positions of the maxima and the asymmetric inhibition patterns of the seven ligands and distamycin on complementary strands.[77]. Asymmetric shifts of the footprints with 3'-skewing is characteristic of the behavior of the minor groove binding agent MPE•Fe(II). Distamycin is used as a reference point for determination of binding site size and shows clearly 5 bp site sizes at both r =0.08 and 0.32. The region 4264-4276 does not afford a long enough AT tract to permit bidentate binding, even for ligand 87 at r =0.08, and at r = 0.32 monodentate

binding covering 5 bp is evident for 77, 78, 86, 87, 89, and distamycin. However, the longer AT-rich tract from 4290-4318 permits effective bidentate binding at r =0.08 for 87 and 89 (corresponding to two of the maxima in Kapp determination) and, at r = 0.32 for 78, 87, and 89(corresponding to all three Kapp maxima). Although it is difficult to assign precise binding site [illegible] or 78, 87, and 89 (n = 2, 4, 6 respectively) the Kapp and footprinting results are in accord with bidentate binding for these even-numbered $(CH_2)_n$ linked agents covering 10±1 bp and which is reflected in their relative binding constants and cytotoxicities (*vide infra*). A summary of the sequence preferential binding of the bis-lexitropsins is given in Table 7.

V. C Cytotoxic Activity

Among the linked bis-lexitropsins tested for cytostatic activity against KB cancer cells (ATCG CCC 17)[76] compound 89 (n = 6) proved to be the most potent with the potency decreasing in the order of 89>78>87>90>77>86≈88. Compound 89 was *ca.* 30% more potent than distamycin (TD_{50} = 8.4 μg/ml) while 78 was comparable with distamycin in potency. The results indicate that compounds 78, 89 and distamycin show significant activity against KB cancer cells and are also active against HSV, MLV, IFZ, and RSV viruses (Table 6). The fact that compounds bearing odd-numbered linkers 77 (n =1), 86 (n =3), 88 (n =5) show relatively lower potencies indicates that the enhanced cytostatic potency observed for the n =2, 4, and 6 compounds is not due to increased lipophilicity as a result of introduction of the $-(CH_2)_n-$ moiety.

V. D Relationship between DNA binding and cytostatic potency: Phasing aspects.

Figure 18 shows a plot of the effects of linker length n on the binding constants (Ka, dotted line) and relative KB cytostatic potency (solid line). A close relationship is evident, taken together with the footprinting results, points to the importance of phasing

Table 7. Preferred binding sequences of distamycin and new compounds deduced from complementary strand MPE•Fe(II) footprinting on EcoRI/Hind III fragment of pBR322 DNA.

	Binding Site								
Compound (n)	(4264-67)	(4273-76)	(4292-95) or (4294-98)	(4296-99)	(4301-04)	(4305-08) or (4304-08)	(4315-18)	(4317-20) or (4315-19)	
Distamycin	ATTT	AAAA	AGAAA			ATTAT			ATTAA
77 (1)		AAAA	TAAG	AAAC	ATTA	TTAT			
78 (2)	ATTT	AAAA	TAAGAAAC		ATTATTATG		ATTA		
86 (3)	ATTT	AAAA	TAAG	AAAC	ATTA	TTAT			
87 (4)	ATTT	AAAA		AAAC	ATTATTATG		ATTA		
88 (5)					ATTA	TTAT			
89 (6)	ATTT	AAAA	TAAGAAAC		ATTATTAT		ATTA	TTAT	
90 (7)			TAAG	AAAC	ATTA	TTAT	ATTA		

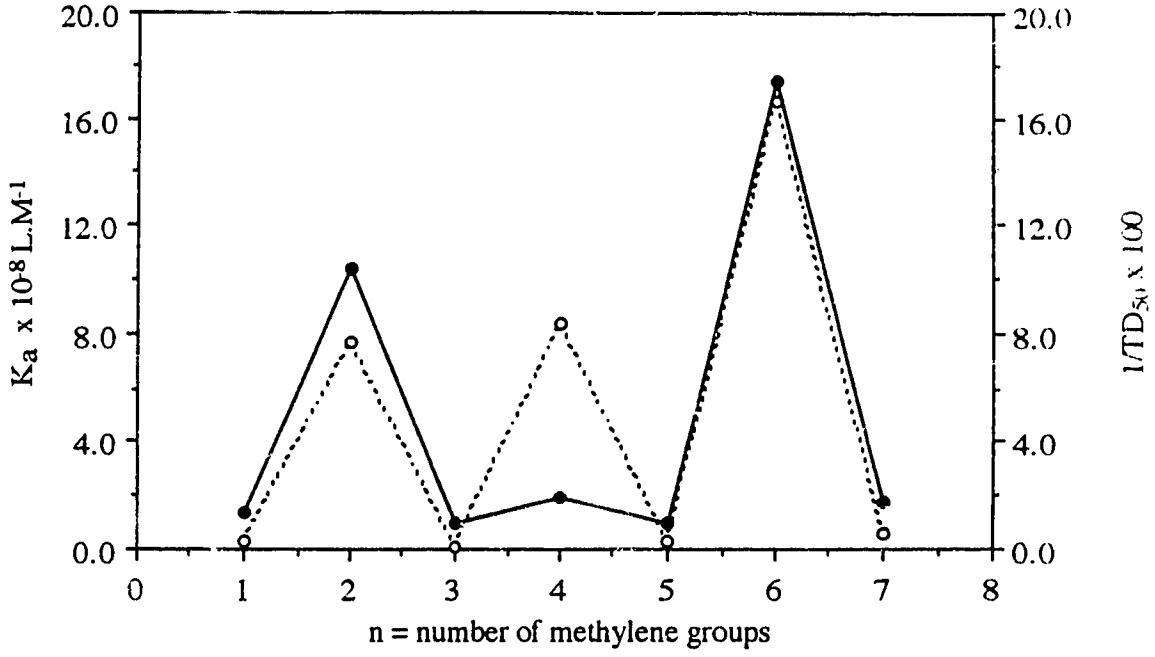


Figure 18. Correlation between DNA binding constant with poly(dA-dT) of methylene linked lexitropsins(dotted line) and cytotoxic activity against KB cells expressed in reciprocal of TD_{50} values(solid line)

which permits effective bidentate binding for n = 2, 4, and 6 that is also reflected in enhanced potency.

VI Discussion

The results indicate that by structural modification especially by the introduction of flexible linkers designed to examine the phasing aspects of DNA binding, cancer cell cytostatic potency can be enhanced for oligopeptide agents based on distamycin or netropsin. These findings permit identification of some structural features that contribute to enhanced potency. DNA binding constant determination shows that the binding of these head-to-tail linked bis-lexitropsins to poly(dA-dT) is either stronger than (n=2, 4, 6) or comparable with (n=1, 3, 5, 7) distamycin. Complementary strand footprinting provides independent evidence of effective bidentate binding within suitable AT-rich tracts covering 10 ± 1 bp for n = 2, 4, and 6 but not for n = 1, 3, 5, and 7 which only engaged in monodentate binding wherein one lexitropsin moiety at a time is bound. The striking feature is that the length of the linker directly dictates binding efficiency. When it corresponds to correct phasing, as was predicted from molecular mechanics and is now confirmed experimentally, bidentate binding is permitted. The correspondence between phasing and binding is much clearer for these head-to-tail bis-lexitropsins than the head-to-head counterparts which show less intrinsic sequence specificities. In the later cases, deviations from modelling predictions were observed for shorter linkers (n = 1, 2, 3) and greater binding was observed for n = 2 than n = 6.[79,80]

Excellent correlation is observed between effective binding and anticancer cytostatic potency against KB cells for this homologous series. The fact that the even-numbered linked agents (n = 2, 4, and 6) are more potent than the odd-numbered homologs (n = 1, 3, 5, and 7) indicates that effective bidentate DNA binding is more important contributor to potency than increased lipophilicity. In conclusion attention to phasing aspects in the

design of MGBA anticancer agents can contribute in a useful and predictable manner to enhanced biological potency.

Chapter 4

Synthesis of Head-to-tail Rigid Linked Bis-lexitropsins

I. Introduction

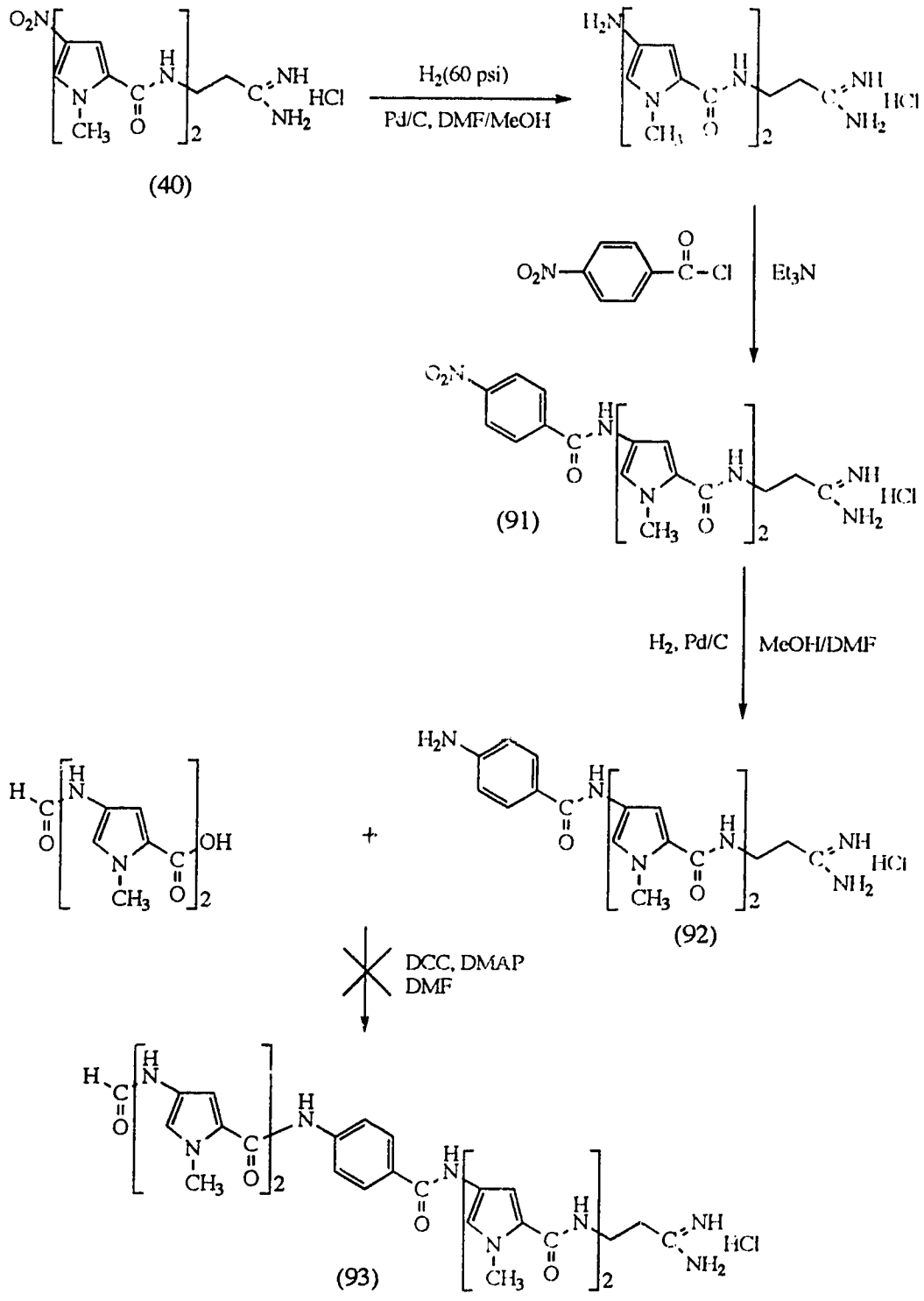
Synthesis of a series of flexible methylene head-to-tail linked bis-lexitropsins was described in chapter 2. Depending on the length of the linkers, either mono- or bidentate binding occurs, which is consistent with and extends the results from flexible head-to-head linked bis-netropsins.[81-82] Bis-netropsins bearing rigid linkers like fumaryl, cyclopropane-dicarbonyl, and p-phenylene dicarbonyl exhibited the highest biological activities.[82] In addition, rigid linked bis-netropsins and bis-distamycin showed consistent higher activity against HIV-1 virus than their flexible linked counterparts.[47] In this chapter, synthetic approachs to some head-to-tail rigid-linked bis-lexitropsins will be described.

II. Description of Experiments

Design and synthesis of head-to-tail rigid linked bis-lexitropsins is based on that of flexible linked oligopeptides described in chapter two. The right and left end parts are (O_2N)-$(Py)_2$-PAH and HCO-$(Py)_2$-OH respectively. Para- and ortho-nitrobenzoic acids were at first employed as linkers. In a preliminary trial, (O_2N)-$(Py)_2$-PAH was reduced to (H_2N)-$(Py)_2$-PAH which was then condensed with p-nitrobenzoyl chloride to give 91 as orange crystals. The nitro group of tripeptide 91 was reduced to amine which, without purification, was coupled with the left end part HCO-$(Py)_2$-OH in the presence of DCC and DMAP (Scheme IX). No new spot was observed on TLC, thus the reaction did not occur because of the low nucleophilicity of amine 92.

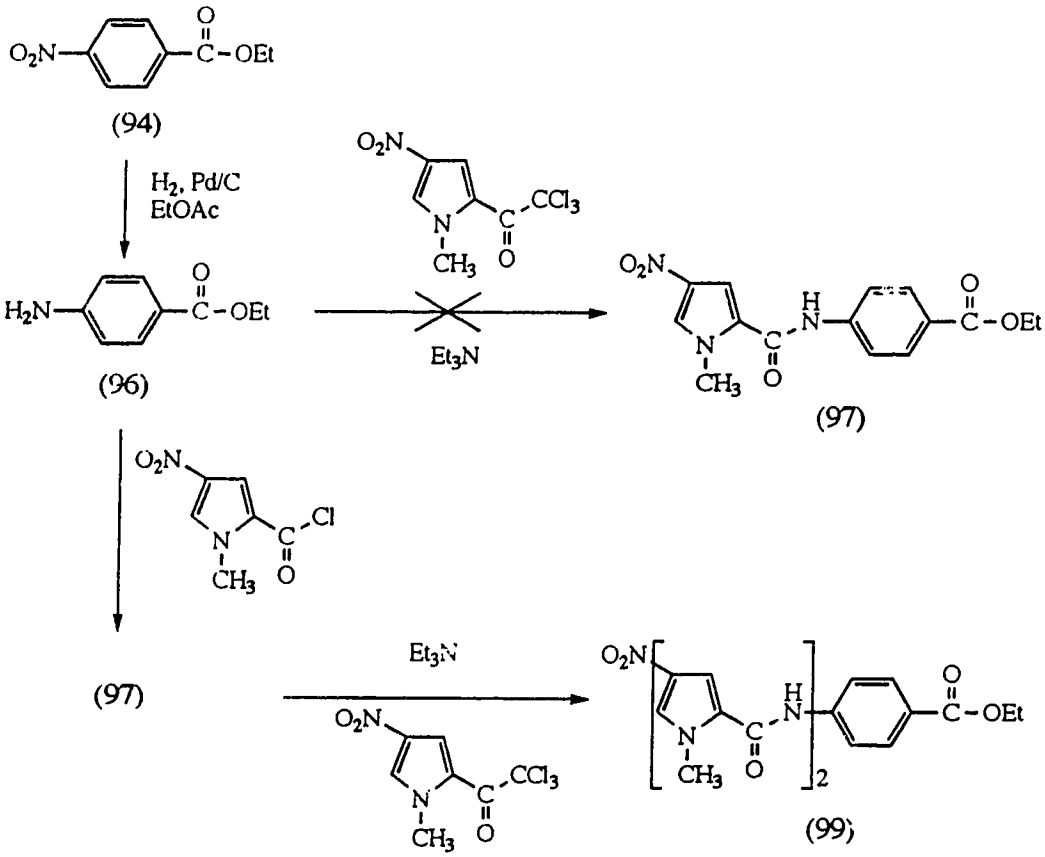
Since the amino group on a benzene ring attached to a electron-withdrawing group is too unreactive to undergo any condensation reaction with carboxylic acid in the presence of

Scheme IX


H2(60 psi)
Pd/C, DMF/MeOH
(40)
Et3N
(91)
H2, Pd/C
MeOH/DMF
(92)
DCC, DMAP
DMF
(93)

activation agents (e. g. DCC and DMAP), an alternative synthetic route as described in chapter II has to be applied. Thus, the carboxyl group of p- or m-nitrobenzoic acid was protected as its ethyl ester; then, the nitro group of ester 94 or 95 was reduced to amine which was condensed with O_2N-Py-CCl_3. The expected product (e. g. 97) did not form, as the deactivated amine 96 is not nucleophilic enough towards O_2N-Py-CCl_3 (Scheme X).

Scheme X

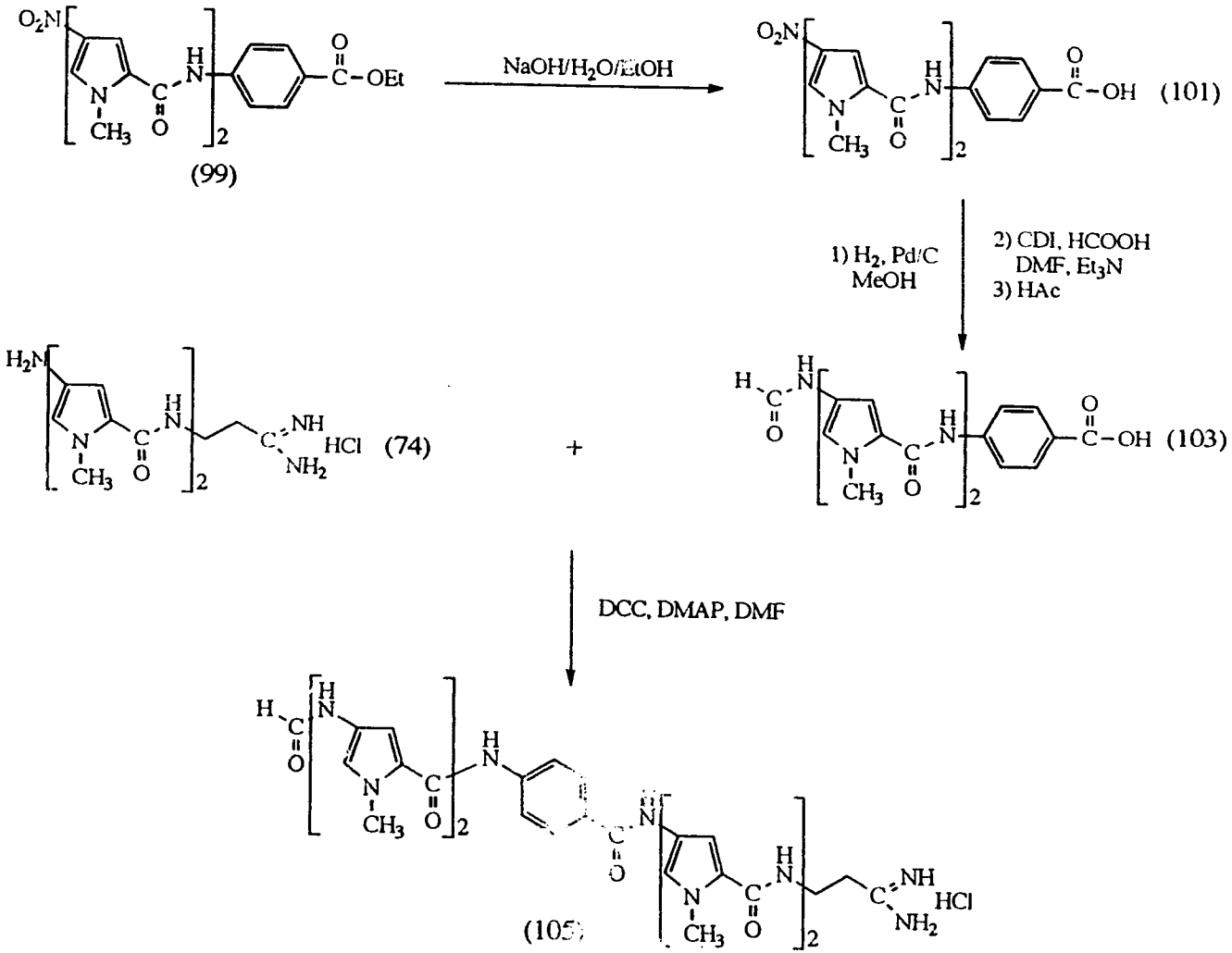


On the other hand, amine 96 reacted with O_2N-Py-Cl in the presence of Hunig's base to give peptide 97 in 86% yield. It was found that both ethyl 3-nitrobenzoate and the corresponding amine were unable to crystallize, therefore, the synthesis of ethyl 3-(1-methyl-4-nitropyrrole-2-carboxamido)benzoate 98 was accomplished without isolation and identification of these intermediate products. The nitro group was reduced to amine which was condensed with O_2N-Py-CCl_3 to form the dipeptide 99. After deprotection of the carboxyl group, the nitro group was reduced to amine which was formylated with a solution (1.5 equivalents) of CDI and HCOOH in THF. Condensation of left portion and the hydrogenated right portion was performed in the presence of DCC and DMAP in DMF to yield the crude lexitropsin 105 which was then purified by reversed-phase HPLC as described in chapter II. Similarly, meta-linked lexitropsin 106 was synthesized (Scheme XI).

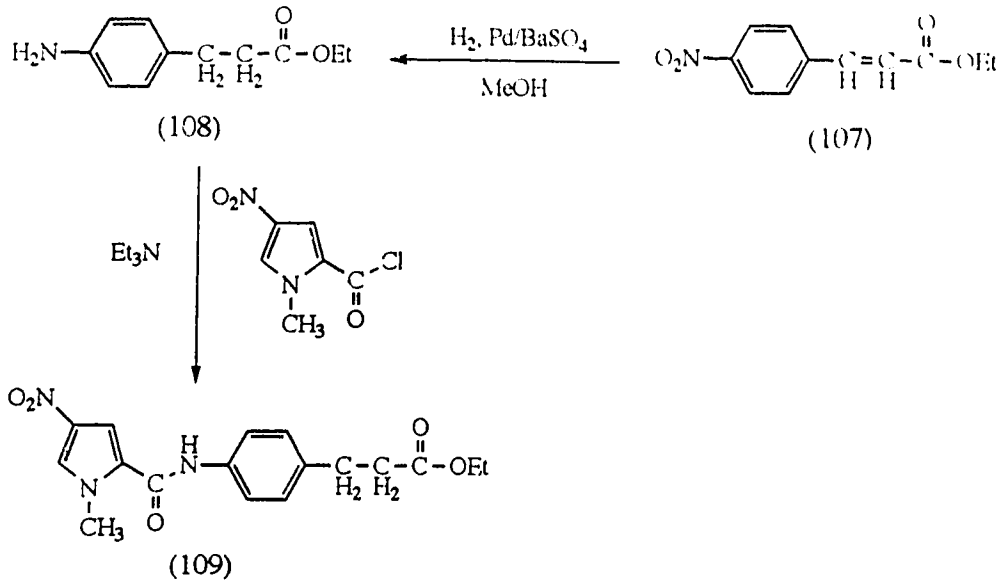
The second group of rigid linkers used in this study were para- and ortho-nitrocinnamic acids. These acids were protected as their corresponding esters as above, then followed by hydrogenation (H_2, 1 atm) using deactivated palladium on charcoal as a catalyst. Both nitro group and double bond were reduced, as nmr spectrum of compound 108 showed no olefinic hydrogens (Scheme XII). It was found later that the conjugation between benzene and alkene facilitate the reduction of the double bond.

The third group of rigid linkers tested was nitrogen containing heterocycles such as 3-amino-1,2,4-triazole-5-carboxylic acid and 6-aminonicotinic acid. The most difficult problem associated with the incorporation of these heterocycles is their very low solubility in any of the solvents. Further investigation of rigid linkers is necessary if any relationship between structure and activity of head-to-tail rigid-linked bis-lexitropsins is to be established.

Scheme XI


NaOH/H2O/EtOH
(99)
(101)
1) H2, Pd/C
MeOH
2) CDI, HCOOH
DMF, Et3N
3) HAc
(74)
(103)
DCC, DMAP, DMF
(105)

Scheme XII


H2N
C-C-C-OEt
H2 H2
O
H2, Pd/BaSO4
MeOH
O2N
C=C-C-OEt
H H
O
(108)
(107)
Et3N
O2N
N
CH3
C
O
Cl
O2N
N
CH3
H
N
C
O
C-C-C-OEt
H2 H2
O
(109)

III. Experimental

Ethyl 4-nitrobenzoate (94) A suspension of p-nitrobenzoic acid (1.68 g, 10 mmol) in thionyl chloride (25 ml) was refluxed on oil bath (90 °C) for 1 h. The excess thionyl chloride was removed in vacuo, and the residue was washed with ether twice and dried. To the solid residue dissolved in THF (10 ml), cooled to -20 °C, a solution of Hunig's base (1.74 ml, 10 mmol), ethanol (5 ml) in THF (5 ml) was added slowly. The mixture was brought up to r.t. and stirring was continued for 4 h. Solvent was removed and the solid residue was dissolved in ethyl acetate, washed with water, filtered through a short column of silica gel, and dried over sodium sulphate. Removal of ethyl acetate gave 94 as a pale solid (1.85 g, 95% yield). Mp: 52-54 °C; lit. mp(Aldrich): 55-59 °C. IR(KBr): 1720, 1520, 1350, 1270, 1100, 720 cm^{-1}. Nmr (DMSO-d_6) δ 1.36(3H, t, J=7.5), 4.38(2H, q, J=7.5), 8.19(2H, d, J=8.0), 8.35(2H, t, J=8.0). FAB-MS: m/z 196(MH^+, 14%).

Ethyl 3-nitrobenzoate (95) The procedure is similar to that above. The product did not crystallize.

Ethyl 4-aminobenzoate (96). A suspension of 10% Pd/C (2g) and ethyl 4-nitrobenzoate (3.98 g, 20.4 mmol) in MeOH (30 ml) was stirred in H_2 (1 atm) for 25 h. The catalyst was removed by filtration and the solvent was evaporated to give a white precipitate. Recrystallization from EtOH/H_2O gave 96 as white crystals (3.18 g, 95% yield). Mp: 89-90 °C; lit. mp (Aldrich): 88-90 °C. Ir (KBr): 3420, 3340, 1680, 1630, 1600, 1370, 1310, 1280, 1170, 1125, 1110, 770 cm^{-1}. Nmr (DMSO-d_6) δ 1.24 (3H, t, J = 7.2), 4.17 (2H, q, J = 7.2), 5.93 (2H, s), 6.54 (2H, d, J = 8.0), 7.61 (2H, d, J = 8.0). FAB-MS: m/z 166 (MH^+, 100%).

Ethyl 4-(1-methyl-4-nitropyrrole-2-carboxamido)benzoate (97). A suspension of 1-methyl-4-nitropyrrole-2-carboxylic acid (0.850 g, 4.97 mmol) in $SOCl_2$

(20 ml) was refluxed until all the solid dissolved. The excess $SOCl_2$ was removed in vacuo and the residue was washed with THF twice. To the amine 96 dissolved in DMF (5 ml), cooled to -20 °C, Hunig's base (0.871 ml, 5 mmol) was added, followed by a solution of 1-methyl-4-nitropyrrole-2-carbonyl chloride in THF (5 ml). The mixture was stirred at r.t. for 18 h, then the solvent was removed. Recrystallization from EtOH gave 97 as pale crystals (1.36 g, 86% yield). Mp: 209-210 °C. Ir (KBr): 3375, 1685, 1600, 1540, 1490, 1410, 1320, 1250, 1180, 770 cm^{-1}. Nmr (DMSO-d_6) δ 1.32 (3H, t, J = 7.5), 3.97 (3H, s), 4.28 (2H, q, J = 7.5), 7.77 (1H, d, J = 1.8), 7.88 (2H, d, J = 8.0), 7.94 (2H, d, J = 1.8), 8.24 (1H, d, J = 1.8), 10.40 (1H, s). FAB-MS: m/z 318 (MH^+, 7.2%).

Ethyl 3-(1-methyl-4-nitropyrrole-2-carboxamido)-benzoate (98). The procedure is similar to that above except that the nitro group of 95 was reduced to amine as that of 94. Yield 83%. Mp: 184-186 °C. Ir (KBr): 3410, 1720, 1670, 1600, 1550, 1530, 1500, 1420, 1320, 1290, 1275, 1120, 1105, 750, 590 cm^{-1}. Nmr (DMSO-d_6) δ 1.33 (3H, t, J = 7.5), 3.98 (3H, s), 4.34 (2H, q, J = 7.5), 7.50 (1H, t, J = 8.0), 7.70 (1H, d, J = 8.0), 7.76 (1H, d, J = 1.8), 8.03 (1H, d, J = 8.0), 8.24 (1H, d, J = 1.8), 8.38 (1H, t, J = 2.0), 10.30 (1H, s). FAB-MS: m/z 318 (MH^+, 8.1%).

Ethyl 4-[1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]benzoate (99). A suspension of 10% Pd/C (0.330 g) and 97 (0.987 g, 3.11 mmol) in MeOH/EtOAc (30 ml/10 ml) was stirred in H_2 (60 psi) on a Paar shaker for 4 h, then filtered. The catalyst was washed thoroughly with MeOH and the combined filtrate and washings were concentrated to a small volume. DMF (2 ml) was added and evaporated. To a solution of this amine dissolved in DMF (5 ml) and cooled to -20 °C, 1-methyl-4-nitro-2-trichloroacetylpyrrole 32 was added in portion. After stirring at r.t. for 18 h, the solvent was removed, ethanol was added, and the resulting precipitate collected. Recrystallization from ethanol gave 99 as yellow crystals (1.09 g, 80% yield). Mp: 270 °C (dec.). Ir (KBr): 3310, 3120, 1660, 1590, 1520, 1410, 1310, 1245, 770 cm^{-1}. Nmr

(DMSO-d_6) δ 1.32 (3H, t, J = 7.5), 3.88 (3H, s), 3.97 (3H, s), 4.28 (2H, q, J = 7.5), 7.23 (1H, d, J = 1.8), 7.36 (1H, d, J = 1.8), 7.61 (1H, d, J = 1.8), 7.92 (4H, m), 8.20 (1H, d, J = 1.8), 10.20 (1H, s), 10.33 (1H, s). FAB-MS: m/z 440 (MH^+, 4.4%).

Ethyl 3-[1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]benzoate (100). The procedure is similar to that above. Yield: 88%. Mp: 223-225 °C. Ir (KBr): 3420, 1710, 1670, 1650, 1550, 1520, 1490, 1320, 1310, 1280, 750 cm^{-1}. Nmr (DMSO-d_6) δ 1.34 (3H, t, J = 7.5), 3.87 (3H, s), 3.96 (3H, s), 4.32 (2H, q, J = 7.5), 7.23 (1H, d, J = 1.8), 7.33 (1H, d, J = 1.8), 7.46 (1H, t, J = 8.0), 7.59 (1H, d, J = 1.8), 7.65 (1H, d, J = 1.8), 8.02 (1H, d, J = 8.0), 8.19 (1H, d, J = 1.8), 8.41 (1H, t, J = 2.0), 10.09 (1H, s), 10.29 (1H, s). FAB-MS: m/z 440 (MH^+, 35%).

4-[1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido] benzoic acid (101). A suspension of 99 (0.775 g, 1.76 mmol) and NaOH (0.353 g, 8.82 mmol) in H_2O/EtOH (40 ml/20 ml) was stirred at 80 °C until all solid dissolved (1.5 h). The hot solution was acidified with concentrated HCl to pH 2, then concentrated, filtered to give yellow precipitate. Recrystallization from EtOH/H_2O gave 99 as yellow crystals (0.550 g, 76% yield). Mp: 330 °C (dec.). Ir (KBr): 3210, 1700, 1660, 1590, 1520, 1505, 1410, 1310, 750 cm^{-1}. Nmr (DMSO-d_6) δ 3.87 (3H, s), 3.97 (3H, s), 7.22 (1H, d, J = 1.8), 7.36 (1H, d, J = 1.8), 7.61 (1H, d, J = 1.8), 7.89 (4H, m), 8.20 (1H, d, J = 1.8), 10.18 (1H, s), 10.32 (1H, s), 12.65 (1H, bs). FAB-MS: no MH^+ peak.

3-[1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]benzoic acid (102). The procedure is similar to that above. Yield 91%. Mp: 317 °C (dec.). Ir (KBr): 3430, 1690. 1640, 1555, 1530, 1500, 1440, 1420, 1320, 1310, 755 cm^{-1}. Nmr (DMSO-d_6) δ 3.87 (3H, s), 3.97 (3H, s), 7.21 (1H, d, J =

1.8), 7.34 (1H, d, J = 1.8), 7.43 (1H, t, J = 8.0), 7.60 (1H, d, J = 1.8), 7.63 (1H, d, J = 1.8), 7.97 (1H, d, J = 8.0), 8.20 (1H, d, J = 1.8), 8.38 (1H, m), 10.07 (1H, s), 10.30 (1H, s). FAB-MS: no MH^+ peak.

4-{1-Methyl-4-[1-methyl-4-(formylamino)pyrrole-2-carboxamido] pyrrole-2-carboxamido}benzoic acid (103). A suspension of 100 (0.550 g, 1.34 mmol), Et_3N (0.205 ml, 1.47 mmol), and 10% Pd/C (0.141 g) in MeOH (25 ml) was stirred in H_2 (60 psi) for 4h. Catalyst was removed by filtration and washed with MeOH, and the combined filtrate and washings were concentrated to a small volume, then DMF (2 ml) was added and evaporated. To a suspension of CDI (0.433 g, 2.67 mmol) in THF (2 ml) was added 98% HCOOH (0.103 ml, 2.67 mmol). After stirring at r.t. for 0.5 h, 1 ml of this solution was added to the amine residue dissolved in DMF (3 ml) and cooled to - 40 °C. Stirring was continued at -40 °C for 45 min, then the solvent was removed in vacuum. MeOH and CH_3COOH was added and the product was precipitated with ether (0.589 g). A small amount of this crude product was purified by reversed-phase HPLC for analytical studies. No distinct melting point. Ir (KBr): 3430, 1650, 1635, 1620, 1600, 1520, 1440, 1400, 1380 cm^{-1}. Nmr (DMSO-d_6) δ 3.86 (6H, s), 6.92 (1H, d, J = 1.8), 7.19 (1H, s), 7.21 (1H, d, J = 1.8), 7.32 (1H, d, J = 1.8), 7.80 (2H, d, J = 8.0), 7.87 (2H, d, J = 8.0), 8.14 (1H, d, J = 1.8), 9.97 (1H, s), 10.06 (1H, s), 10.10 (1H, s), 12.65 (1H, bs). FAB-MS: calcd. mass for $C_{20}H_{19}N_5O_5$, 410.1464; found via exact mass FAB, 410. 1449 (1.3%).

3-{1-Methyl-4-[1-methyl-4-(formylamino)pyrrole-2-carboxamido] pyrrole-2-carboxamido}benzoic acid (104). The procedure is similar to that above. No distinct mp. Ir (KBr): 3280, 1650, 1590, 1540, 1440, 1400, 1250, 1200, 755 cm^{-1}. Nmr (DMSO-d_6) δ 3.87 (6H, s), 6.93 (1H, d, J = 1.8), 7.20 (1H, d, J = 1.8), 7.21 (1H, d, J = 1.8), 7.31 (1H, d, J = 1.8), 7.41 (1H, t, J = 8.0), 7.61 (1H, d, J = 8.0), 7.95 (1H, d, J = 8.0), 8.13 (1H, s), 8.38 (1H, m), 9.96 (1H, s), 10.02 (1H, s),

10.07 (1H, s). FAB-MS: calcd. mass for $C_{20}H_{19}N_5O_5$, 410.1464; found via exact mass FAB, 410. 1443.

1-Methyl-4-{1-methyl-4-{4-{1-methyl-4-[1-methyl-4-(formylamino) pyrrole-2-carboxamido]pyrrole-2-carboxamido}benzamido}pyrrole-2-carbcxamido}pyrrole-2-carboxamidopropionamidine hydrochloride (105). A suspension of amidine 40 (0.304 g, 0.764 mmol) and 10% Pd/C (0.090 mg) in DMF/MeOH (10 ml/ 10 ml) was stirred in H_2 (60 psi) on Paar shaker apparatus for 4h. The catalyst was collected and washed with MeOH, and the combined filtrate and washings were concentrated to a small volume. To the amine residue dissolved in DMF (2 ml) and cooled to 0 °C, acid 103 (0.313 g, 0.764 mmol), DMAP (0.093 g, 0.764 mmol), and DCC (0.158 g, 0.764 mmol) was added sequentially. The solution was brought up to r.t. and the stirring was continued for 18 h. Solvent was removed, MeOH was added, followed by ether, and the resulting precipitate was collected (0.502 g). Purification by reversed-phase HPLC gave analytically pure solid with an overall yield of 6% from 101. No distinct mp. Ir (KBr): 3420, 1650, 1635, 1540, 1520, 1440, 1405 cm^{-1}. Nmr (DMSO-d_6) δ 2.56 (2H, t, J = 6.0), 3.48 (2H, m), 3.81 (3H, s), 3.84 (3H, s), 3.86 (6H, s), 6.925 (1H, d, J = 1.8), 6.94 (1H, d, J = 1.8), 7.08 (1H, d, J = 1.8),), 7.21 (1H, d, J = 1.8), 7.215 (1H, d, J = 1.8), 7.225 (1H, J = 1.8), 7.32 (1H, d, J = 1.8), 7.33 (1H, d, J = 1.8), 7.87 (2H, d, J = 8.0), 7.93 (2H, d, J = 8.0), 8.13 (1H, s), 8.26 (1H, t, J = 5.4), 9.96 (1H, s), 9.975 (1H, s), 10.09 (1H, s), 10.10 (1H, s), 10.22 (1H, s). FAB-MS: calcd. mass for $C_{35}H_{38}N_{12}O_6$ (M-Cl), 723.3115; found via exact mass FAB, 723.3091 (3.2%).

1-Methyl-4-{1-methyl-4-{3-{1-methyl-4-[1-methyl-4-(formylamino) pyrrole-2-carboxamido]pyrrole-2-carboxamido}benzamido}pyrrole-2-carboxamido}pyrrole-2-carboxamidopropionamidine hydrochloride (106). The procedure is similar to that above. Overall yield from 102 is 8%. No distinct mp. Ir (KBr): 3420, 1650, 1640, 1590, 1540, 1440, 1400 cm^{-1}. Nmr (DMSO-d_6) δ 2.57 (2H,

t, J = 6.0), 3.48 (2H, m), 3.82 (3H, s), 3.87 (3H, s), 3.89 (6H, s), 6.92 (1H, d, J = 1.8), 6.93 (1H, d, J = 1.8), 7.085 (1H, d, J = 1.8),), 7.21 (1H, d, J = 1.8), 7.215 (1H, d, J = 1.8), 7.22 (1H, J = 1.8), 7.31 (1H, d, J = 1.8), 7.34 (1H, d, J = 1.8), 7.44 (1H, t, J = 8.0), 7.60 (1H, d, J = 8.0), 7.91 (1H, d, J = 8.0), 8.13 (1H, s), 8.27 (1H, m), 9.97 (2H, s), 10.07 (1H, s), 10.10 (1H, s), 10.35 (1H, s). FAB-MS: calcd. mass for $C_{35}H_{38}N_{12}O_6$ (M-Cl), 723.3115; found via exact mass FAB, 723.3087 (1.29%).

Conclusions and Prospects

Our goal to explore the structural features that contribute to the DNA sequence selectivity and anticancer potency of the minor groove binding agents has led to the design and synthesis of a series of head-to-tail linked bis-lexitropsins. It has proven instructive in that it has revealed a clear correspondence between phasing and binding of lexitropsins to the double-stranded DNA. From this work, the following conclusions have been reached.

(1) 1-Methyl-4-nitro-2-trichloroacetylpyrrole is a versatile precursor for the synthesis of oligo-N-methylpyrrole carboxamide antibiotics like lexitropsins.

(2) Bis-tri-n-butyltin oxide is shown to be useful in the preparation of peptide acids O_2N-Pyr-A_xa in that it both protects the C-terminus and converts the N-terminus of the ω-amino acids to a good nucleophile.

(3) Reversed-phase HPLC is a beneficial tool for the purification of high polar, H_2O/MeOH soluble lexitropsins; ammonium acetate is a critical buffer in that it provides better resolution, maintains the pH of the eluent neutral, and is readily removed *via* freeze-drying.

(4) Synthesis of head-to-tail polymethylene-linked bis-lexitropsins has been achieved. The binding of these new bis-lexitropsins to poly(dA-dT) is either stronger than (n = 2, 4, 6) or comparable (n = 1, 3, 5, 7) with that of distamycin. Effective bidentate binding occurs within suitable AT-rich tracts covering 10 ± 1 bp for n = 2, 4, 6, but not for n = 1, 3, 5, 7.

(5) The anticancer cytostatic potency against KB cell for this homologous series indicates that effective bidentate binding is a more important contributor to potency than increased lipophilicity.

(6) Synthesis of two head-to-tail rigid-linked bis-lexitropsins have been described. The KB cell cytotoxicity assay indicates moderate potency, while footprinting studies are underway.

The intriguing finding that head-to-tail linked bis-lexitropsins with n = 6 is the most potent agent among this homologous series indicates that bis-lexitropsins with n > 7 should be explored. Further investigation of the relationship between structure and activity of head-to-tail rigid-linked bis-lexitropsins may prove beneficial according to the results from the head-to-head linked counterparts. The fact that the new series of lexitropsins with appropriate phasing cover up to 10 ± 1 bp within a suitable AT-rich tract of double helical DNA implies the possibility of 16 bp binding if tridentate lexitropsins are applied.

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