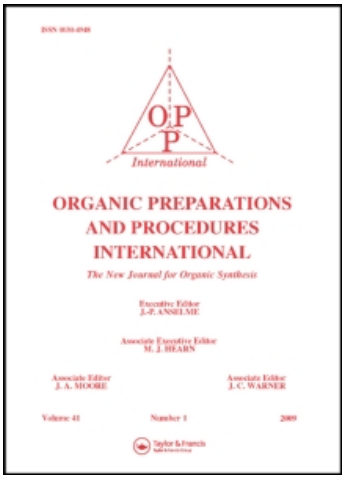


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# SYNTHETIC CHEMISTRY OF NATURALLY OCCURRING OLIGOPEPTIDE ANTIBIOTICS AND RELATED LEXITROPSINS

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# SYNTHETIC CHEMISTRY OF NATURALLY OCCURRING OLIGOPEPTIDE ANTIBIOTICS AND RELATED LEXITROPSINS

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## INTRODUCTION

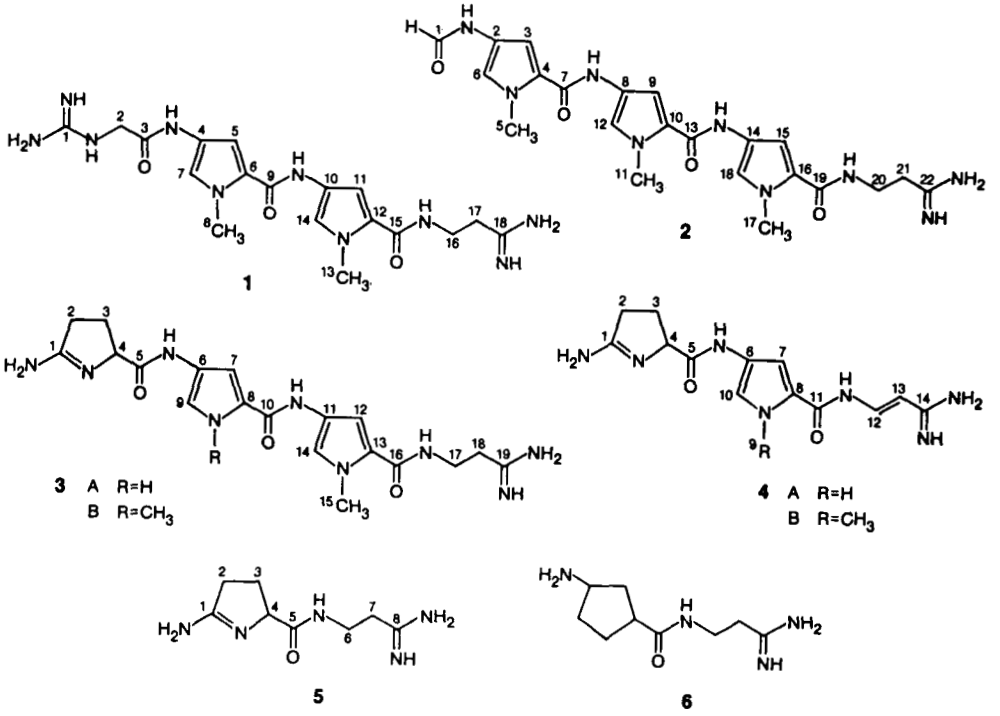
The thriving and creative field of synthetic organic chemistry continues to respond to challenges presented in biology. Among the more interesting problems that are beginning to be addressed is that of molecular recognition between biomolecules. This widespread phenomenon has important consequences in many biological processes including, enzyme catalysis and inhibition, gene transcription, replication, recombination, repair or in transposition, the immunological response, transport phenomena and drug action.[1,2] Considerable insight has been gained into enzyme action by the judicious selection and design of enzyme model systems.[3a,m,4] Similarly the concept of host-guest molecular interactions has added significantly to our knowledge of specificity of action.[2] A no less important and related problem, which is central to molecular biology and molecular genetics, is that of control of information. This question is important enough and wide enough to motivate half or more of the research going on in molecular biology today, from development and differentiation to genetic engineering, and the study of oncogenes.[5]

At the heart of this problem is the necessity to elucidate the protein-DNA molecular recognition code.[6] At present our understanding of the determining structural factors in this type of molecular recognition is primitive. Control proteins and xenobiotics appear to utilize two different channels of information.[6,7,8] The former, exemplified by promoters repressors and many enzymes, in general employ the more information-rich major groove,[6b,7,8] although there is evidence for important molecular contacts between e.g. DNase I and some polymerases with the minor groove.[9] In general non-intercalative small molecule xenobiotics recognize and bind to the minor groove.[10] Thus insight into some of the contributing factors may be gained by studying natural and synthetic DNA sequence specific agents.



The paradigm of naturally occurring non-intercalative sequence specific agents is netropsin, **1**[11a] a member of a modest group of pyrrole-amidine oligopeptides which includes distamycin A, **2**;[12] anthelvencins, **3**;[13] kikumycins, **4**;[14] amidinomycin, **5**;[15] and noformycin, **6**[16] (Scheme 1), and which exhibit antibiotic, antiviral and antitumor activity.

Several lines of evidence from a study of their biochemical pharmacology indicate that they act to block the template function of DNA by binding to specific nucleotide sequences in the minor groove of double helical DNA.[10,12b] These sequences are $(AT)_4$ and $(AT)_5$ respectively for netropsin and distamycin A. Examination of the structural requirements for this specific molecular recognition deduced, in part, from recent X-ray studies on the complex of netropsin with a dodecamer,[17] suggested that replacement of one or more pyrrole moieties by a hydrogen bond accepting heterocycle, such as imidazole, should result in the rational alteration of base recognition from AT to GC (Figure 1). This reasoning gave rise to the concept of lexitropsins, or information-reading oligopeptides, described below.[18]




**Scheme 1**. Structures of naturally occurring oligopeptide antibiotics: **1**, netropsin; **2**, distamycin; **3**, anthelvencins A and B; **4**, kikumycins A and B; **5**, amidinomycin; **6**, noformycin.

The sine qua non of this particular approach to the development of DNA sequence reading agents is efficient, general and adaptable synthetic procedures to both the naturally occurring oligopeptide antibiotics and the related lexitropsins. The purpose of this review is to describe the progress and some of the obstacles encountered in such syntheses to date.

## I. SYNTHESIS OF NETROPSIN

Netropsin **1** was isolated from *Streptomyces netropsis* by Finlay et al. in 1951,[19] and synonyms are congocidin[20] and sinanomycin.[21] Its structure was elucidated by Waller et al. in 1957 and it was shown to belong to the pyrrole-

amidine group of antibiotics which exhibit antibacterial, antiviral and antitumor activity.[22] Netropsin has a dramatic therapeutic effect on experimental vaccinia virus infection in mice but is without action against infections from RNA viruses.[23] Netropsin is also effective against Rauscher virus-induced lymphoid leukemia in mice.[24] The antibiotic evidently acts as an inhibitor of RNA and DNA biosynthesis which can be attributed to the strong binding of the antibiotic to DNA.[10,12b] Netropsin, like the other oligopeptide antibiotics is unstable in solution.

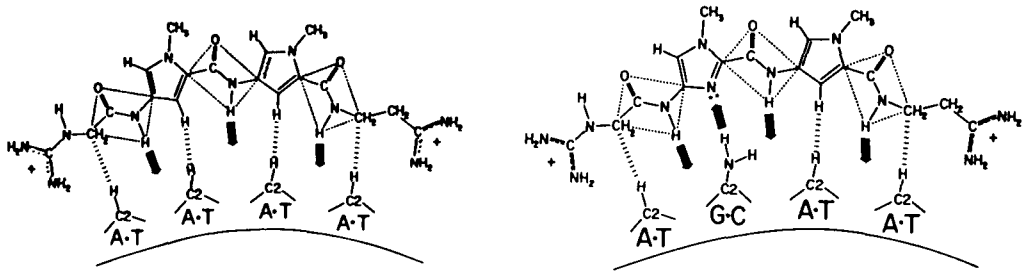



**Figure 1.** Idealized molecular contacts with DNA sequences by (a) netropsin, (b) an imidazole containing lexitropsin.



General Considerations of Synthetic Strategy

A number of total syntheses of natural oligopeptide antibiotics have been reported.[11,12a,25-27] All except one[27] have been based essentially on the method introduced for the synthesis of netropsin by Julia and Preau-Joseph.[11] The strategy consisted of introducing the amidine group early, carrying forward the nitro-heterocyclic intermediate which is then reduced catalytically prior to coupling of the guanidine-acetyl group.[11] Some variations in the preparation of the intermediate nitropyrrole derivatives were introduced by Bialer[26] and by Grehn and coworkers.[27] The existing methods do not lend themselves to the development of an adaptable general synthetic procedure required for the synthesis of a variety of oligopeptide agents. These methods also suffer from several disadvantages

including (i) the coupling reactions employ unsatisfactory methods e.g. the reaction of an amine with an acyl chloride in the presence of aqueous sodium bicarbonate or triethylamine in ethanol and give unsatisfactory yields: one procedure also uses uncommon protecting groups for coupling,[27] (ii) existing methods of introduction of the formyl group in distamycin[25-27] are unsatisfactory with regard to yield and the required purification procedure, (iii) the sequence of attachment of the end groups is inconvenient in that the products require column chromatography which is undesirable because of contamination with inorganic salts (from the adsorbents) of the final oligopeptides that are customarily isolated as hydrochloride salts, (iv) the reaction conditions for the Pinner reaction were not optimized.

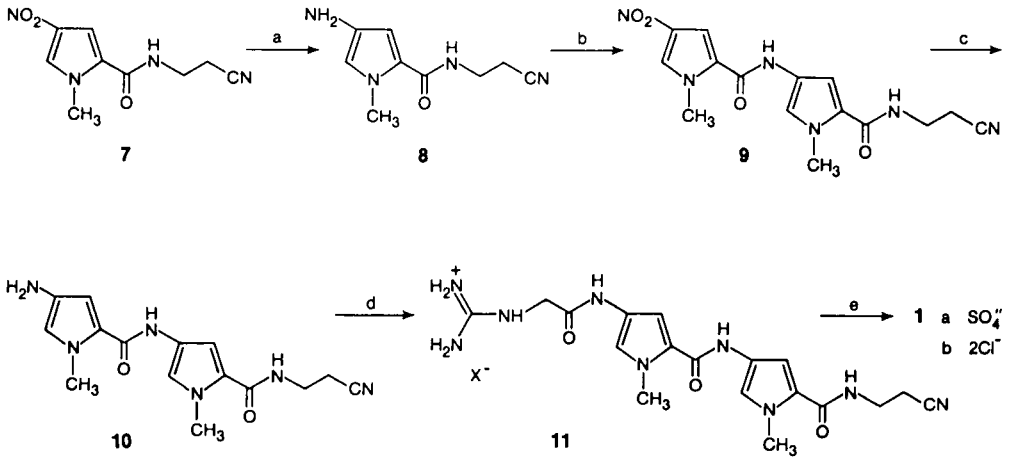
Thus our objectives were to try to improve existing methods, not only increasing the yields, but also to avoid chromatographic methods of separation.



Synthesis of Netropsin

Nitration of 1-methyl-2-pyrrole carboxylic acid[26] or that of its ethyl ester[27] gives a mixture of the desired 4-nitro derivative and other nitro derivatives.[28] Existing methods employ column chromatography for the separation of the nitro esters.[26] We found that when 1-methyl-2-pyrrole carboxylic acid is nitrated with nitric acid:acetic anhydride mixture the 4-nitropyrrole acid crystallizes from the reaction mixture at low temperature as a pure isomer in fair yield of 43%. Consequently the 1-methyl-4-nitropyrrole acid may be obtained directly and in a procedure that avoids chromatography. The acid was converted into its chloride with thionyl chloride and coupled with aminopropionitrile in the presence of Hunig's base to give **7** in 95% yield (Scheme 2). The latter was reduced by catalytic hydrogenation to give the amine **8** which was isolated as the hydrochloride in 46% yield. The free amine of **8** in common with the other 4-aminopyrroles is extremely unstable. In contrast the corresponding hydrochloride salt is more stable and may be stored and consequently was used in some steps of our syntheses. Hydrogenation of **7** to **8** is almost quantitative and **8** was used as formed immediately without isolation.

Acylation of the 4-amino group in **8** with the 4-nitropyrrole acid chloride afforded **9** in 91% yield. The yield of the latter was improved to 95% by acylating **8** directly without isolation as the hydrochloride (Scheme 2).




**Scheme 2.** Reaction conditions: (a), $H_2$, Pd/C in MeOH; (b) 1-methyl-4-nitro-imidazole-2-acylchloride and $iPr_2EtN$ in THF; (c), $H_2$, Pd/C in MeOH; (d), guanidineacetic acid hydrochloride, DCC in DMSO then $Na_2SO_4$; (e), HCl in EtOH, then dry $NH_3$, EtOH then $Na_2SO_4$, $BaSO_4$ gives **1a**.

The nitro compound **9** was reduced catalytically to give the amine **10**, which is stable in the solid form but not in solution. The amine **10**, when allowed to react with guanidineacetic acid in the presence of dicyclohexylcarbodiimide afforded **11** isolated as the sulfate. Pinner reaction[29] on **11** using HCl in ethanol, followed by ammonia gave an excellent yield (77%) of netropsin **1a** isolated as the sulfate. We found that the reaction of the nitrile group with ethanol in the presence of HCl is completed after 90 min and that longer reaction times give more side products and decomposition.[30] In addition reaction of the iminoether with $NH_3$ is almost instantaneous and there is no need to run the reaction overnight as previously claimed.[30]

Our procedure introduces the oligopeptide end groups in the reverse order from that reported hitherto.[11] Among the advantages are that compound **11** can be

readily isolated in pure form and, since the last step is virtually quantitative by TLC examination, the final product **1a** is almost pure. Compound **1a** was then treated with barium chloride to obtain the corresponding hydrochloride **1b**, the form in which netropsin was originally isolated.[11] In order to avoid contamination of **1b** with barium chloride, a slight excess of **1a** was used and the hydrochloride **1b** was extracted with methanol in which **1a** is insoluble. The netropsin hydrochloride **1b** thus obtained was identical with an authentic sample of the antibiotic.

## II. SYNTHESIS OF DISTAMYCIN

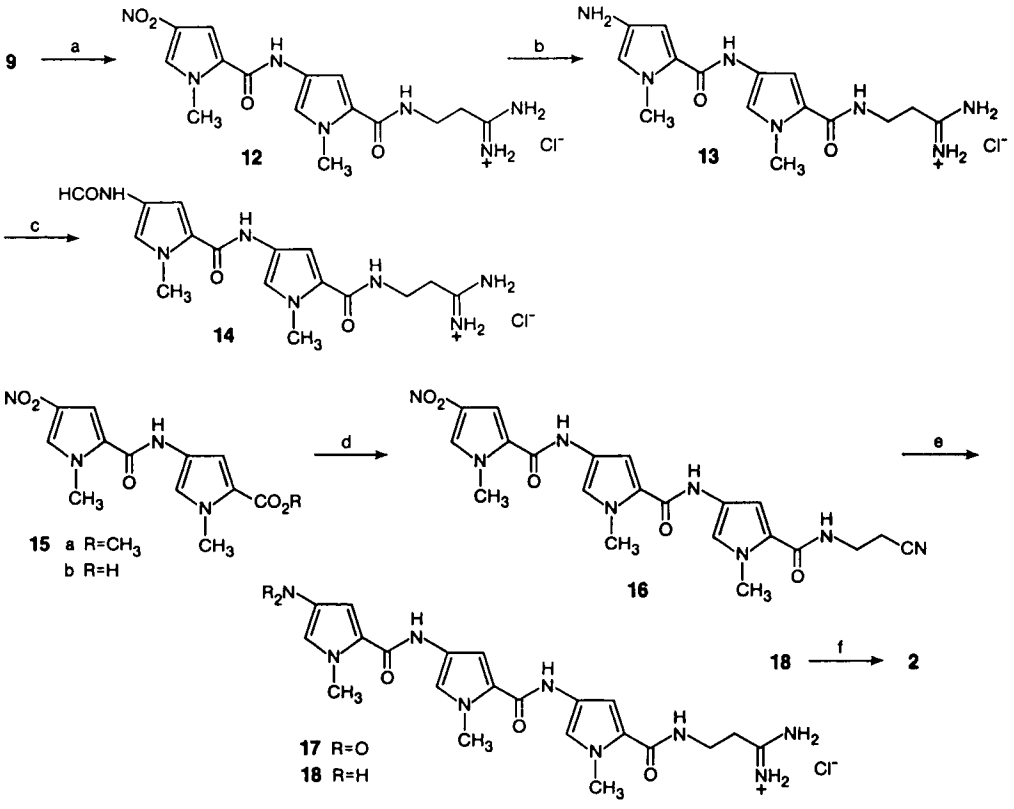
Distamycin A is a fermentation product of *Streptomyces disticallus*.[31] The chemical structure of distamycin A **2** has been established by organic chemical methods.[25,32] Distamycin A like its congenors in fermentation mixtures, exhibits antifungal activity.[32] The antibiotic has significant effects on ascites tumors such as Ehrlich and S 180 in mice. It also causes marked decrease in the growth of solid tumors, Ehrlich adenocarcinoma, sarcoma 180, Walker carcinoma and Oberling-Guerin-Guerin myeloma.[33] The most important antiviral effects of distamycin A are directed against DNA-containing viruses.[12b] In man, distamycin A ointments have been used successfuly for the topical treatment of chickenpox, herpes zoster and eruptions resulting from smallpox vaccination.[34-38] Distamycin A acts as a DNA template poison and inhibits the cell-free synthesis of RNA by RNA polymerase.[39] It has been shown that the antibiotic inhibits preferentially the initiation of RNA synthesis rather than the subsequent elongation phase.[40] This specific effect is attributed to an occupancy of A-T rich regions of template DNA which are known to exist at the initiation sites of RNA transcription.[40] Of considerable biological significance may be the ability of distamycin to inhibit the DNA polymerase reaction catalyzed by an enzyme of the RNA-containing Rous sarcoma virus or of murine sarcoma virus.[41,42]

In order to adapt our procedure for netropsin for the synthesis of distamycin an efficient means of introducing the terminal N-formyl group was necessary.

N-formylation has been the least satisfactory step in all the synthetic procedures developed for distamycin and related structures. Acetic-formic anhydride,[25] formic acid and dicyclohexylcarbodiimide,[26] or formamide and ethyl formate[43] rarely give yields up to 40% and rather more often are in the range 10-20%. An additional disadvantage has been the necessity to purify the product by column chromatography.[26,27] Pinner reaction on **9** afforded **12** which was reduced catalytically to **13**.

The amino compound **12** and **1a** were found to be unstable in the presence of formic acid (accounting for the poor yields of some previous methods[25,26]) so a milder neutral method of N-formylation was sought.[28] We examined Staab's reagent N-formylimidazole for this purpose.[44] When the reagent is pure it is very hygroscopic and unstable. However we found that it can be used without isolation and it reacts with amino heterocycles such as **13** or **1a** in methanolic solution to give a quantitative yield of the N-formyl product as judged by TLC examination. The excess of the reagent reacts with methanol to give volatile methyl formate and imidazole the latter of which is readily removed by extraction with nonpolar solvents. This efficient method was then applied to the synthesis of distamycin.

Starting from the nitropyrrole ester the nitro-dipyrrole peptide ester **15a** was prepared using the chemistry described previously. Hydrolysis of the ester and condensation of **15b** with compound **8** in the presence of a proton sponge and DCC afforded **16** in 64% yield. This procedure has the advantage of avoiding DCC in the introduction of the side chain with the attendant problems of separation and reduced yield resulting from side reaction of DCC with the amine.[26] Application of our modified Pinner reaction conditions gave the amidine **17.** Catalytic reduction of **17** gave the stable amine **18** in 70% yield. Treatment of the amine **18** with N-formylimidazole gave pure distamycin **2** in 71% yield identical with an authentic sample (Scheme 3).



**Scheme 3.** Reaction conditions: (a), HCl in EtOH, then dry $NH_3$, EtOH; (b), $H_2$, Pd/C in MeOH; (c), N-formylimidazole in THF; (d), aqueous-ethanolic NaOH, heat, then compound **8** hydrochloride, 1,8-bis-dimethylamino-naphthaline in DMA, then DCC; (e), HCl, EtOH, then dry $NH_3$, EtOH, $H_2$ Pd/C in MeOH; (f), N-formylimidazole in THF/MeOH.

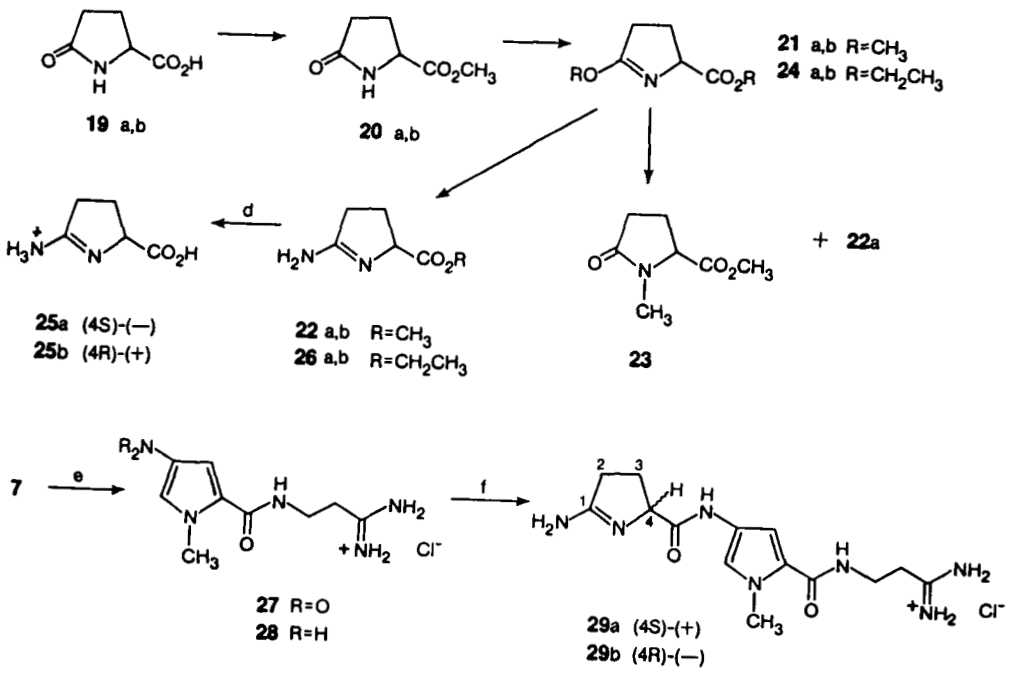
These polar peptidic antibiotics containing guanidinyl and/or amidine groups are readily analyzed for purity by TLC on silica gel with methanol as eluent. Acetic acid is necessary as a cosolvent when only one strongly polar group is present and formic acid is used when two polar groups are present in the molecule. For such polar compounds FAB-MS[45] proved satisfactory for determining the molecular

composition. When one polar group is present peaks corresponding to $(M\text{-}HSO_4)^+$, $(M\text{-}Cl)^+$, $(2M\text{-}Cl\text{-}HCl)^+$ and $(2M\text{-}Cl)^+$ are detected. When two polar groups are present in the molecule the ions $(M\text{-}HSO_4)^+$, $MH^+$, $(2M\text{-}HSO_4)^+$, $M_2H^+$ are detected for sulfates and $(M\text{-}Cl\text{-}HCl)^+$ and $(M\text{-}Cl)^+$ for hydrochlorides.

## III. SYNTHESIS OF (4S)-(+)-DIHYDROKIKUMYCIN B AND ITS (4R)-(-) ISOMER AND PROOF OF ABSOLUTE CONFIGURATION OF NATURAL ANTIBIOTICS

The structures of the antibiotics kikumycin A and B **4** and their dihydro-derivatives were established in 1972.[14] It is clear that these agents belong to the group of pyrrole-amidine antiviral antibiotics which includes netropsin and distamycin.[11,12] The kikumycins differ from the former however by possessing a chiral center in common with the anthelvencins **3** (*vide infra*). They are produced by *Streptomyces phaeochromogenes* R-719.[14b] Kikumycins A and B are effective against gram-positive and gram-negative organisms including *Actinomyces bovis* in a concentration of 6-25 mcg/mL.[14b] While they are structurally related to netropsin and distamycin A, no antiviral nor antitumor activities have been detected to date with kikumycins.[14b] Until now there has been no reported synthesis of these antibiotics. The presence of the chiral center poses a challenge synthetically and raises interesting questions with respect to the effects of chirality on molecular recognition. The strategy was to develop an efficient synthesis of the 2-amino-1-pyrroline synthon, and subsequent coupling to an aminopyrrole unit leading to the convergent syntheses of (+)- and (-)-dihydrokikumycin B, **29a** and **29b** respectively. The approach employed for the syntheses of both enantiomers of dihydrokikymycin B, as depicted in Scheme 4, requires the preparation of the 2-amino-1-pyrroline moiety and the pyrrole unit separately, then coupling of these two groups should afford the carbon skeleton of **29a** and **29b**.

The chiral starting materials for the direct enantioselective syntheses of **29a** and **29b** are the methyl esters **20a,b** which could be prepared readily from




**Scheme 4.** Reaction conditions: (a), $SOCl_2$, MeOH, DMF, room temperature; (b), $(R_3O)BF_4$, $CH_2Cl_2$, room temperature; (c), $NH_4Cl$, MeOH, heat; (d) 10% HCl, 50°C, 2-5 h; (e), HCl in dry EtOH, then dry $NH_3$, then $H_2$, Pd/C, MeOH, room temperature; (f), **25a**, DCC, DMAP, DMF, room temperature.

(S)-(-)-**19a** and (R)-(+)-**19b**, respectively.[46] Acid **19b** can be readily synthesized by refluxing (R)-(-)-glutamic acid in water.[47] The first step in our approach to prepare **29a** and **29b** is the transformation of the 2-pyrrolidone moiety in **20a** and **20b** respectively, into the corresponding 2-amino-1-pyrroline moiety by way of an imidate ester.[48] Both **29a** and **29b** are prepared according to the procedures given in Scheme 4; however, only the synthesis of **29a** will be described in detail. According to a recently published procedure, treatment of **20a** with dimethyl sulfate gave the methyl imidate ester **21a**.[49] However, subsequent treatment of **21a** with ammonium chloride in refluxing methanol gave the desired product **22a** in low

yield (20%). The major product obtained is the N-methyl pyrrolidone **23** (40%) which is presumably formed from tautomerization of the methyl group in **21a**.

We anticipated that the ethyl imidate ester would be more stable towards rearrangement.[50] Accordingly, treatment of **20a** with Meerwein salt (triethyl-oxonium tetrafluoroborate)[51] gave the desired ethyl imidate ester **24a** in 95% yield. The presence of the imidate ester moiety in **24a** is confirmed by the imine stretch at 1643 $cm^{-1}$ in the IR spectrum. Ester **24a** was converted with ammonium chloride in refluxing methanol to afford the intermediate **22a** in almost quantitative yield. The composition of **22a** is established by the appearance of the base peak at m/z 2143 for the M-Cl fragment. In addition, the presence of the amidine group in **22a** is confirmed by the appearance of an amidine stretch at 1682 $cm^{-1}$ in the IR spectrum, and three exchangeable protons at δ9.88, 9.55 and 9.21 ppm in the $^1$H-NMR spectrum. $^1$H-NMR analysis of ester **22a** in the presence of a chiral shift reagent (tris[3-trifluoromethyl-hydroxymethylene)-(+)-camphorato], europium III) shows about 5% racemization for the overall transformation from **19a**. Next, the ester moiety in **22a** was hydrolyzed in aqueous hydrochloric acid (10%) to give acid **25a** in high yield (Scheme 4).[52]



The pyrrole unit required for the formation of **29a** can be readily synthesized from 1-methyl-4-nitropyrrole-2-carboxylic acid.[28] Nitration of the latter followed by coupling with β–aminoethyl cyanide gave nitrile **7**.[28] Pinner reaction of **7** (HCl in ethanol, then ammonia)[29] gave amidine hydrochloride **27** in high yield.[43] The nitro group in **27** was reduced catalytically to give amine **28**, which when allowed to react with acid **25a** in the presence of dicyclohexylcarbodiimide (DCC) gave (4S)-(+)-dihydrokikumycin B in a moderate yield of 55%. The optical purity of (4S)-(+)-**29a** determined by $^1$H-NMR in the presence of a chiral shift reagent is 80 ± 4 percent enantiomeric excess. These conversions allow the 4S configuration to be assigned to the product (+)-**29a**. Since the dextrorotatory dihydrokikumycin B

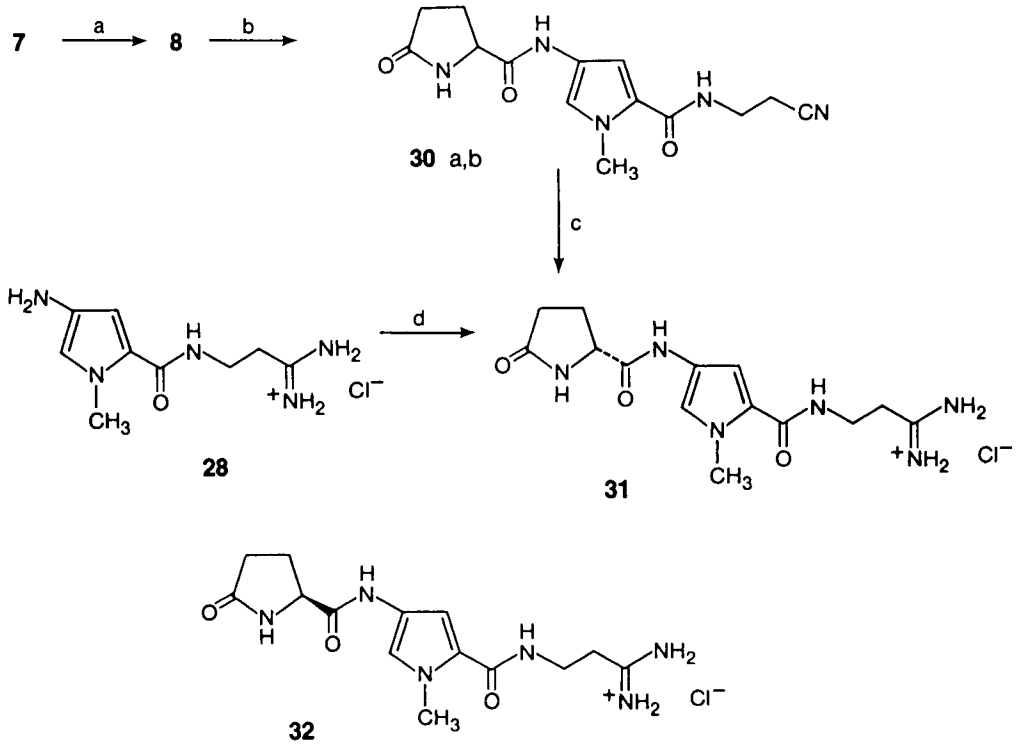
**29** is derived directly from the natural antibiotic kikumycin B **4** the latter is accorded the (4S)-(+) absolute configuration.

We were also interested in comparing the DNA binding ability of the 2-amino-1-pyrroline moiety in **29a,b** to their pyrrolidone analogues **31** and **32**. Our initial attempt to prepare **31** was based on the approach to the synthesis of noformycin by Diana,[16] and it is depicted in Scheme 5. Hydrogenation of the nitro-compound **7** over palladium on charcoal gave the unstable amine intermediate **8** which was directly coupled with acid **19a** to give pyrrolidone **30** in good yield (71%). However, Pinner reaction on **30** gave a complex product mixture, and analysis of the $^1$H-NMR spectrum of the crude material showed only a small amount of the desired product **31** (judged by the intensity of the low field amide NH signal at 10.1 ppm). An alternative route to prepare **31**, would be to condense amine **28** with acid **19a**, since this method would avoid the problematic Pinner reaction step as previously encountered with **30**. Accordingly, reaction of amine **28** with **19a** in the presence of DCC gave (+)-**31** in moderate yield (50%). The enantiomeric (-)-**32** was prepared similarly (Scheme 5).



Sequence Specificity of Binding of Enantiomeric Dihydrokikumycins to DNA

The sequence specific binding of (4S)-(+)-dihydrokikumycin B and its (4R)-(-) enantiomer, (**29a** and **29b**, respectively) to DNA were determined from DNase I and MPE footprinting and $^1$H-NMR studies.[53] Footprinting analyses showed that both enantiomers [(+)- and (-)-**29b**] bind to AT-rich regions of DNA, with occasional GC base pairs allowed. The dihydrokikumycins, while recognizing similar DNA sequences to those preferred by the tripeptide antibiotics, should require a shorter binding site.[54,55] While binding strongly to the $(A.T)_3$ site present in the plasmid DNA fragment, the ligands also interact to a significant degree with sequences containing GC base pairs. Evidently the smaller dihydrokikumycins are less discriminating than the tripeptide antibiotics.




**Scheme 5.** Reaction conditions: (a), $H_2$, Pd/C, MeOH, room temperature; (b), **25a** or **25b**, DCC, DMAP, DMF, room temperature; (c), HCl in dry EtOH, then dry $NH_3$, EtOH; (d), DCC, DMAP, DMF, room temperature.

Based on the MPE footprinting data, the chiral center of dihydrokikumycin B clearly plays a role in defining binding selectivity to DNA, with the natural (4S)-(+) isomer binding somewhat more efficiently to DNA. $^1$H-NMR studies (ligand induced chemical shift changes and NOE differences) of the dihydrokikumycins with d-[CGCAATTGCG]$_2$ show unambiguously that the N to C termini of the ligands are bound to 5'-$A_5T_6T_7$-3' reading from left to right. From quantitative 1D-NOE studies, the AH2(5)-ligand H7 distance of complex A [(4S)-(+)-**29a** plus decamer which is bound more strongly] and complex B [(4R)-(-)-**29b** and decamer] are estimated to be 3.83Å and 4.94Å, respectively.[52] This difference in binding properties is reflected in the thermodynamic profiles of the two enantiomeric ligands derived from microcalorimetry. The binding free energy ($\Delta G°$) of (+)-**29a**

and (-)-**29b** to poly d(AT).poly d(AT) at 25°C are -15.1 and -13.0 kJ $mol^{-1}$, respectively.[53] Collectively these data provided models for the binding processes of the enantiomeric dihydrokikumycins and insight into the influence of chirality on the molecular recognition processes and provides an additional component in the design of DNA sequence specific agents.

## IV. SYNTHESIS AND PROOF OF ABSOLUTE CONFIGURATION OF (4S)-(+) ANTHELVENCIN A AND ITS (4R)-(-) ENANTIOMER

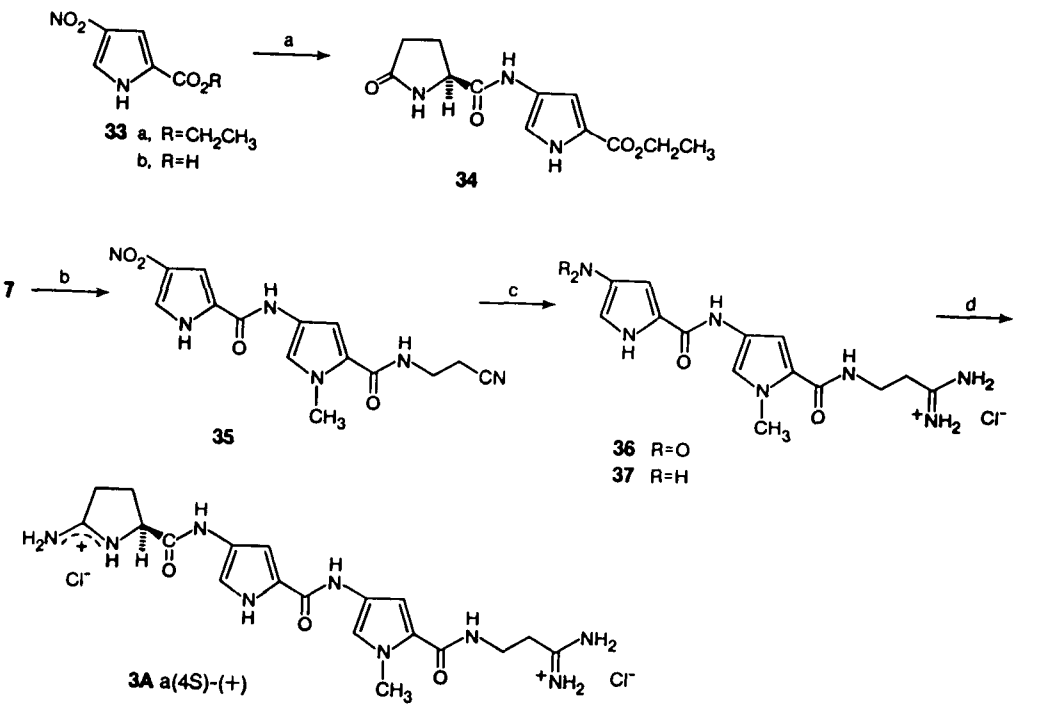
Anthelvencins A and B **3** are additional naturally occurring oligopeptides isolated from the culture *Streptomyces venezuelae.*[13] Anthelvencin has modest activity against a wide spectrum of bacteria administered subcutaneously or intraperitoneally in mice including E. coli, *proteus vulgaris* and *Klebsiella pneumoniae.* In swine fed with anthelvencin (12 g/ton of food) clearance of 96% of the total population of *Ascoris suis,* 78% of oesophagostomum, and 100% of *Trichuris suis* is observed with no toxicity in a 56 day test.[13] Oral administration to rats of 180 mg/kg of anthelvencin salicylate controlled *Entamoeba histolytica.* In tissue cultures anthelvencin has some activity against vaccinia virus.[13] As in the case of the kikumycins neither the absolute configuration of the anthelvencins nor their DNA sequence specificity had been established. Our objectives therefore included the development of a total synthesis of both enantiomeric forms, establishment of the absolute configuration of the natural product, and investigation of the influence of chirality on the DNA sequence recognition.

The synthetic route to anthelvencin A **3a** is outlined in Scheme 6 and follows our recent synthesis of (+)- and (-)-dihydrokikumycin **29b**.[48] In the synthesis of anthelvencin A, a 4-aminopyrrole-2-carboxylate synthon is needed. The required starting nitro-pyrrole ester **33**,[56] can be readily prepared from glycine ethyl ester hydrochloride and sodium nitromalonic aldehyde.[57] Only a limited amount of information is available on the condensation of β-aminopyrroles with carboxylic acid derivatives.[58] This has been, in part, due to the limited availability of β-

aminopyrroles.[58] Therefore, we have examined the condensation of (4S)-(-)-2-pyrrolidone-5-carboxylic acid with the β-aminopyrrole derived from catalytic reduction of ester **33a**.[59] This condensation reaction was effected with dicyclohexylcarbodiimide (DCC) and a catalytic amount of 4-dimethylaminopyridine (DMAP) to afford ester **34** in 71 percent yield. The coupling reaction did not proceed without DMAP, and only tar was obtained. This is presumably because of the low nucleophilicity of the aminopyrrole combined with its high instability (Scheme 6).

Alkaline hydrolysis of ester **33a** followed by acidification provided acid **33b** in quantitative yield.[56] Nitrile **7** can be readily prepared in high yield from 1-methyl-2-pyrrolecarboxylic acid.[28] Catalytic reduction of the nitro substituent in **7** provided an unstable amine intermediate which was coupled with the acid chloride of **33b**[60] in the presence of triethylamine to afford **35** in 70 percent yield. Pinner reaction of **35** with HCl in dry ethanol,[29] followed by treatment of the imidate ester intermediate with ammonia gave the amidine hydrochloride **36** in quantitative yield. The presence of the amidinium moiety in **36** is confirmed by the appearance of two exchangeable $^1$H-NMR signals (two protons each) at 8.78 and 9.10 ppm, and the disappearance of the infrared nitrile stretch of **35** at 2247 cm$^{-1}$. The nitro group of **36** was reduced catalytically to give amine **37**, which is unstable in solution. The amine **37**, when allowed to react with the optically active (4S)-(-)-2-amino-1-pyrroline-5-carboxylic acid **25a**[48] (84 ±4 percent e.e.) in the presence of DCC and DMAP gave (+)-anthelvencin A **3Aa** in 49 percent yield. The structure of **3Aa** is confirmed by the presence of a M-H-2Cl ion at m/z 428 in the FAB-MS analysis, the appearance of the $^1$H-NMR NH3 signal at 10.52 ppm, and by comparison with the published IR and UV data of the natural product.[13]

$^1$H-NMR analysis of **3Aa** in the presence of a chiral shift reagent (tris[3-trifluoromethylhydroxymethylene)-(+)-camphorato], europium III) in a 1:1 DMSO-$dc_6$:$CD_3CN$ solution, and measurement of the resulting unequal




**Scheme 6.** Reaction conditions: (a), $H_2$ Pd/C, MeOH, room temperature, then **19a** or **19b**, DCC, DMAP, DMF, room temperature; (b), $H_2$, Pd/C, MeOH, room temperature, then acid chloride of 4-nitropyrrole-2-carboxylic acid, $Et_3N$ in THF; (c), HCl in dry EtOH, then dry $NH_3$, then $H_2$, Pd/C, MeOH, room temperature; (d), DCC, DMAP, (4S)-(-)**19a** or (4R)-(+)-**19b**, DMF, room temperature.

diastereotopic amide NH5 signals at about 10 ppm provided the enantiomeric excess (e.e.) of **3Aa** to be 80 ± 4 percent. Therefore, judging by the small differences in e.e. values of **3Aa** (80 ± 4%) and the starting acid **25** (84 ± 4%), racemization in the coupling reaction effected by DMAP is minimal, i.e. ~2%. Comparison of the specific rotation of the synthetic material **3Aa** with that of the natural antibiotic[13] establishes the absolute configuration of (+)-anthelvencin as (4S). The unnatural (4R)-(-)-anthelvencin **3Ab** was synthesized using the procedure described above from acid (4R)-(+)-**25**.

We have also attempted to prepare both enantiomers of anthelvencin B **3**.[13] Reduction of the nitro moiety in **12**[28] followed by coupling with acid (4S)-(-)-**25a** gave **3B**. However, **3B** is highly hygroscopic and unstable, thus a pure sample of anthelvencin B cannot be obtained. This high instability may be the reason why the nor-methyl form of **3B** (i.e. the natural product **3A**) is selected for by the producing microorganism.[13]

DNA Sequence Specificity of Natural (+) Anthelvencin A and Its Enantiomer

The influence of the chiral center of anthelvencin A [the natural (4S)-(+) enantiomer **3Aa** and the unnatural isomer (4R)-(-)-**3Ab**] on the binding specificity, affinity and dynamic properties to the decadeoxyribonucleotide d-[CGCAATTGCG]$_2$ were studied by $^1$H-NMR spectroscopy as well as by DNase I footprinting.[61] The drug-induced chemical shifts and NOE measurements of the 1:1 complex of (4S)-(+)-**3Aa** and (4R)-(-)-**3Ab** to the decadeoxyribonucleotide [complexes A and B, respectively] reveal that both enantiomeric forms of anthelvencin A bind in the minor groove along the sequence 5'-$A_4A_5T_6T_7$-3' of the DNA. The site of binding on the duplex as determined using $^1$H-NMR is in agreement with the footprinting evidence.. NOE studies show that the (4S)-enantiomer **3Aa** is propeller twisted about the between the pyrrole units to provide the structural flexibility for the drug to bind snugly in the minor groove along the 5'-AATT sequence on the DNA in complex A.

From molecular modeling studies (Figure 2), it is shown that the (4S) hydrogen of **3Aa** is directed out of the minor groove thereby making the positively charged 2-amino-1-pyrrolinium moiety positioned for favorable electrostatic interaction between the drug and DNA. Conversely, for (4R)-**3Ab** of complex B, the positively charged 2-amino-1-pyrrolinium group is directed out of the minor groove and as a result the interaction of this group with the negative potential of that section of the DNA is restricted. In addition, the conformation of the drug:AATT component of the two complexes are different. The natural (4S)-enantiomer **3Aa** is not bound

centrally between the d-[$A_4A_5T_6T_7$).($A_4A_5T_6T_7$)] DNA duplex but is located diagonally across the 5'-AATT base pair sequence. In contrast, the unnatural (4R)-enantiomer **3Ab** behaves like achiral lexitropsins, and binds centrally between the 5'-AATT base pair sequence. The rate of exchange of the chiral lexitropsins between equivalent sites is found to be dependent on the chirality of the oligopeptides. The exchange rate of (4S)-**3Aa** and (4R)-**3Ab** between the two equivalent 5'-AATT sites via the intramolecular flip-flop mechanism are ~36 $s^{-1}$ at 21°C with $\Delta G^{\ddagger}$ of 68.3 ± 5 kJ $mol^{-1}$ and ~77 $s^{-1}$ at 21°C with $\Delta G^{\ddagger}$ of 62.5 ± 5 kJ $mol^{-1}$, respectively. Compounds **3Aa** and (4R)-**3Ab** bind to poly d(AT).poly d(AT) with $\Delta G$ values of -17.15 and -16.73 kJ/mole respectively at 25°C.



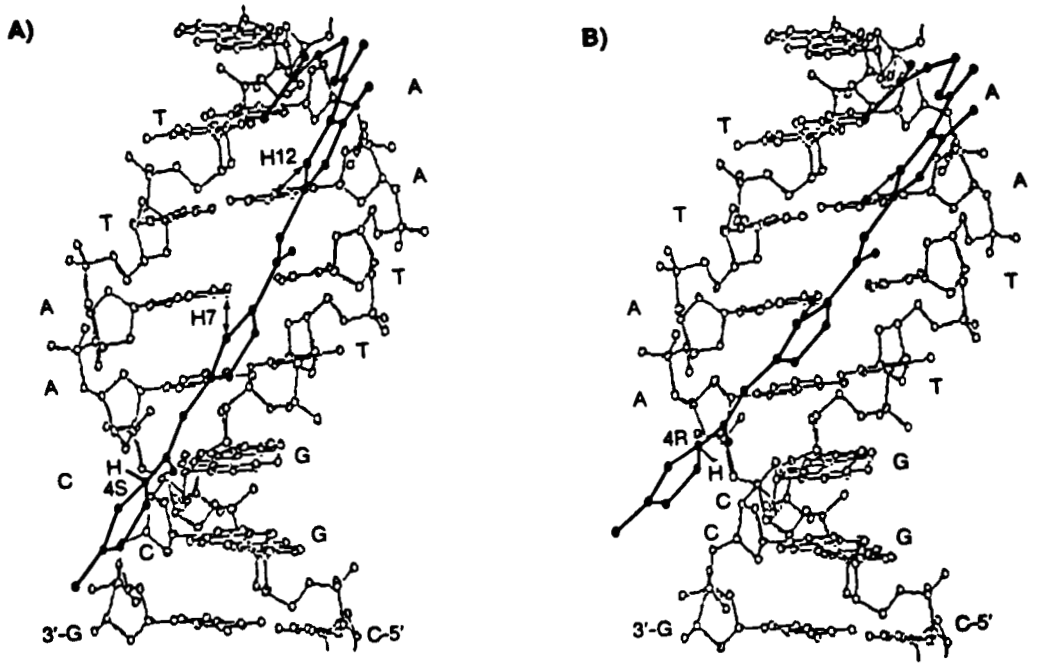



**Figure 2.** Computer generated depiction of sequence specific DNA binding of (A) natural (4S)-(+)-anthelvencin A and (B) unnatural (4R)-(-)-anthelvencin A. The double headed arrow represents the NOE observed between H7 and H12 of the drug and AH2(5) of the DNA.

## V. SYNTHESIS OF PROTOTYPE LEXITROPSINS BASED ON NETROPSIN

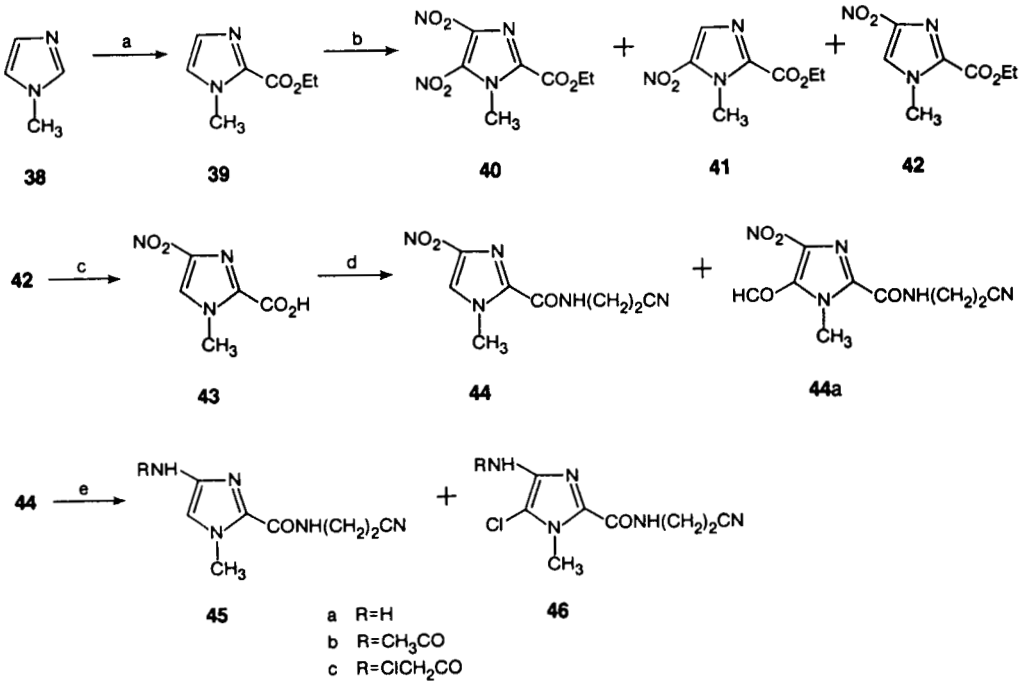
Examination of the structural requirements for the molecular recognition of netropsin for DNA, deduced, in part, from recent X-ray studies on a complex of the antibiotic with a dodecamer,[17] suggested that the replacement of one or more pyrrole rings by imidazole, or other appropriate heterocycle, should result in a rational alterataion of base recognition from AT to GC.[18] The latter prediction follows from the formation of new hydrogen bonds between G-(2)-$NH_2$ in the minor groove and the N3 of the imidazole moiety. The anticipated need to examine a number of new structures in order to establish the molecular recognition characteristics for DNA binding required development of an efficient and flexible general synthesis. The syntheses of the imidazole analogues[62] are essentially based on our method of synthesis of netropsin[28] and distamycin[28] but with modifications necessitated by the presence of imidazole moieties.

Nitration of 1-Methylimidazole-2-carboxylic Acid

Reaction of 1-methylimidazole with ethyl chloroformate in the presence of triethylamine afforded the ester **39**.[63] Nitration of **39** afforded three products **40**, **41**, and **42** which were readily separable and of which only the 5-nitro derivative **41** has been described previously [64] (Scheme 7). Development of this reaction showed that the highest yield of the desired compound **42** is obtained using a 1:1 mixture of 100% nitric acid and sulfuric acid at a temperature of 95° for 50 min. Longer reaction times tend to give more of the undesired 4,5-dinitro derivative **40**. Reaction of **39** with a mixture of nitric acid and acetic anhydride is very slow whereas a similar reaction using trifluoroacetic anhydride is very rapid and affords largely the dinitro compound **40**. Compound **42** was isolated from the preferred procedure in crystalline form in 46.5% yield. Alkaline hydrolysis of **42** afforded the

imidazole-2-carboxylic acid **43** after acidification in 95% yield. Compound **43** proved to be sensitive to decarboxylation upon heating under acidic conditions.

The initial attempt to condense the acid **43** with aminopropionitrile employed oxalyl chloride which however afforded, in addition to the main product **44** in 80% yield, a small amount of the 5-formyl derivative **44a** (Scheme 7). The latter plausibly arises from initial oxylation at position 5 followed by hydrolysis of the acyl chloride and decarboxylation. For this reason an alternative procedure was adopted employing the mixed anhydride of **43** and pivalic acid to give **44** in 91% yield (Scheme 7).



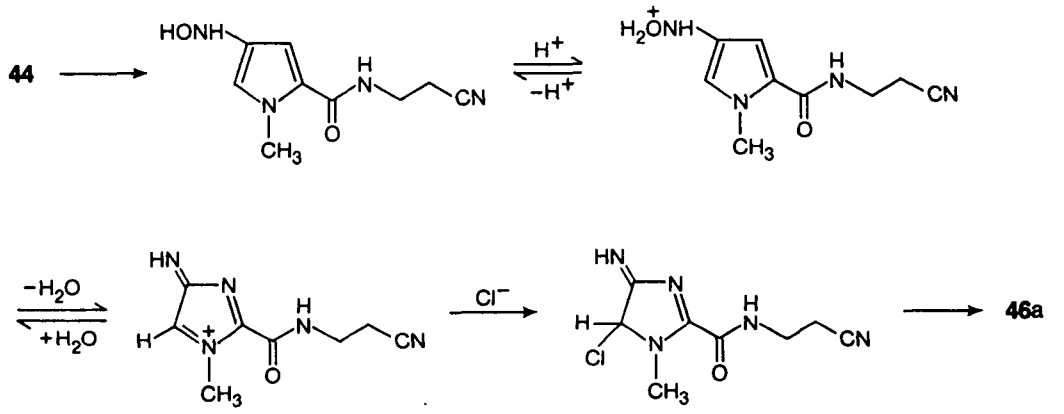

**Scheme 7.** Reaction conditions: (a), $ClCO_2Et$, $Et_3N$; (b), $H_2SO_4$, $HNO_3$; (c), aq. NaOH, then HCl; (d), $(COCl)_2$ or pivaloyl chloride and $NEt_3$, add $NH_2(CH_2)_2CN$; (e), $SnCl_2 \cdot 2H_2O$ + HCl, acetyl chloride, chloroacetyl chloride or acyl chloride of **43**.

Reduction of Nitroimidazole Derivatives with Stannous Chloride

Since catalytic reduction of nitroheterocycles was inconvenient on a larger scale an alternative procedure was investigated. Reduction of **44** with stannous chloride afforded a mixture of two compounds **45a** and **46a** which were not separated but were identified by means of their N-acetyl and N-chloroacetyl derivatives **45b**, **46b** and **45c** and **46c** respectively (Scheme 7). The compositions of the derivatives **45(b,c)**, **46(b,c)** were established unequivocally by MS exact mass measurement.

In the case of chloroacetyl derivatives **45c** and **46c** the ratio of 5-chloro derivative to the nonchlorinated one was 56:44. However, this ratio depends on the order of addition of the reactants, being higher if $SnCl_2$ is added slowly to the nitro compound dissolved in aq. HCl, and lower if the nitro compound is added to the solution of $SnCl_2$ in aq. HCl. Coupling of the compounds **45a** and **46a** (as a mixture) with the acid chloride of **43** gave the corresponding peptides **47** and **48** (Scheme 9) which were readily isolable in 50% and 12% yields, respectively, and which were identified individually. Stannous chloride reduction of compound **48** also led to two products **49** and **50** which were readily separated by chromatography to give 30% and 57% yields respectively. No such chlorination of the second imidazole ring of **48** occurs so this reaction is evidently connected with the process of reduction of the nitro group.

A large excess of stannous chloride caused a decrease in the proportion of chlorination products from about 56% to about 2%. However this cannot be due to reduction of the chloro substituent by stannous chloride, similar to that described by Rinkes[65] because the chloro derivatives **46** and **50** survive such prolonged treatment. We also found that neither compounds **45a** nor **50** react with stannous chloride. We suggest that this 5-imidazole chlorination may follow the mechanism in Scheme 8, which is analogous to that of the reaction of N-phenylhydroxylamine with HF affording p-aminofluorobenzene[66] and that of the reduction of



**Scheme 8.** Possible mechanism for the 5-chlorination of imidazole residues in oligopeptides by $SnCl_2$.

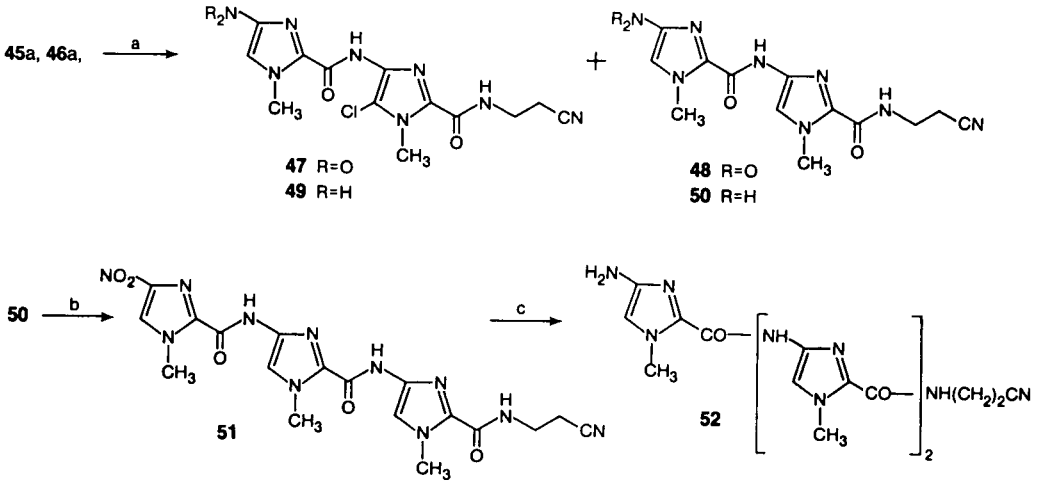


nitrobenzene with stannous chloride and hydrochloric acid producing a mixture of 53% of chloraniline and 47% of aniline.[67] Small amounts of chloro derivatives formed in the presence of a large excess of $SnCl_2$ means that in this case the reduction of the intermediate hydroxylamine group prevails over the rearrangement.

Although the reduction of the nitro compounds with stannous chloride affords some of the side product due to imidazole chlorination, it was faster and more convenient on a larger scale for intermediate stages than catalytic reduction in the presence of palladium. However, in order to avoid chlorination in the step leading to the final three ring compound **51** the nitro compound **48** was reduced catalytically with hydrogen in the presence of palladium on charcoal to give **50** in an excellent yield of 80% (Scheme 9). Coupling of the dipeptide **50** with the acid chloride of **43** afforded the tripeptide **51** in 68% yield. Similar catalytic reduction of **51** gave the amino tripeptide **52** in 65% yield (Scheme 9).

Introduction of the Guanidineacetyl Moiety

With the bisimidazole dipeptide **50** and the corresponding trisimidazole tripeptide **52** in hand we now turned to the attachment of the guanideneacetyl end

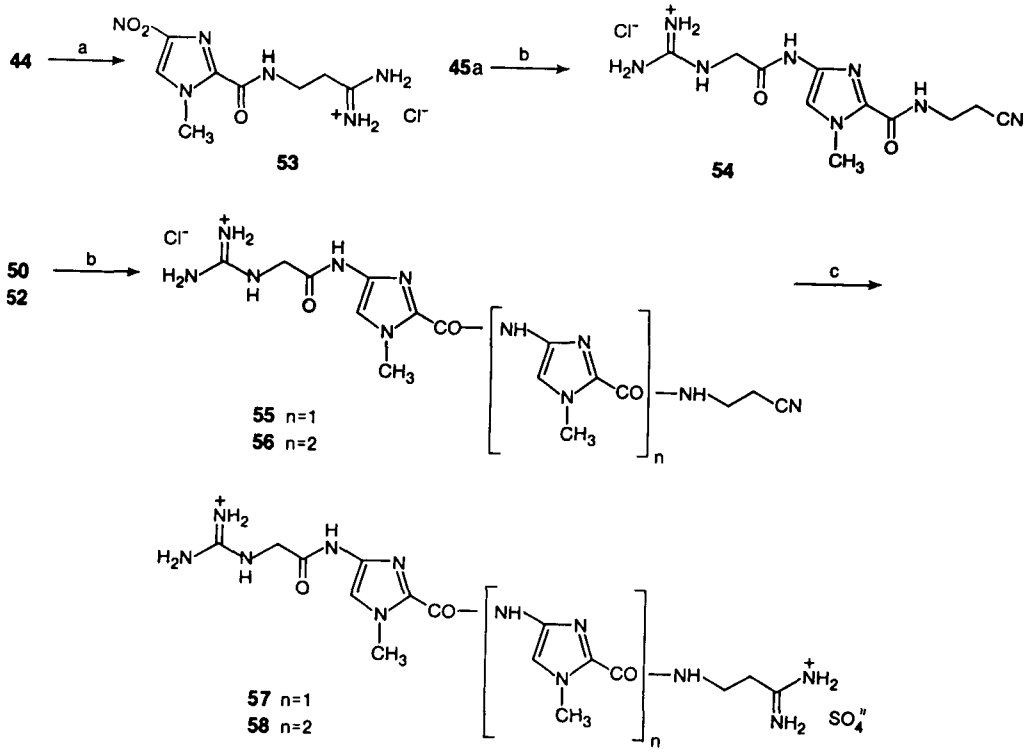



**Scheme 9.** Reaction conditions: (a), $H_2$ Pd/C, in MeOH, room temperature, then acid chloride of **43**, $Et(iPr)_2N$, then $H_2$, Pd/C, in MeOH; (b), acid chloride of **43**, $Et(iPr)_2N$; (c), $H_2$, Pd/C in MeOH.

group. The existing literature method of coupling the amine with guanidineacetic acid hydrochloride in the presence of dicyclohexylcarbodiimide (DCC) was unsatisfactory because of the low yield caused by a side reaction between DCC and aminopyrrole derivatives.[68] Therefore alternative procedures were examined. Attempted couplings of the chloroacetyl group in **45** with guanidine carbonate, acetate or free base were unsuccessful. A report on cyanaminoacetic acid formation from disodium cyanamide and chloroacetic acid[69] suggested an alternative method.[70] Therefore reactions with model compounds and sodium cyanamide were investigated, which were however unsuccessful. Therefore we reexamined the condensation in the presence of DCC by optimizing the conditions. The best results were obtained using one equivalent of the nitro compound e.g. **44**, **48**, or **51** to be reduced, 1.5 equivalents of guanidineacetic acid hydrochloride and 1.5 equivalents of DCC.

Introduction of the Amidine Moiety by Modified Pinner Reaction

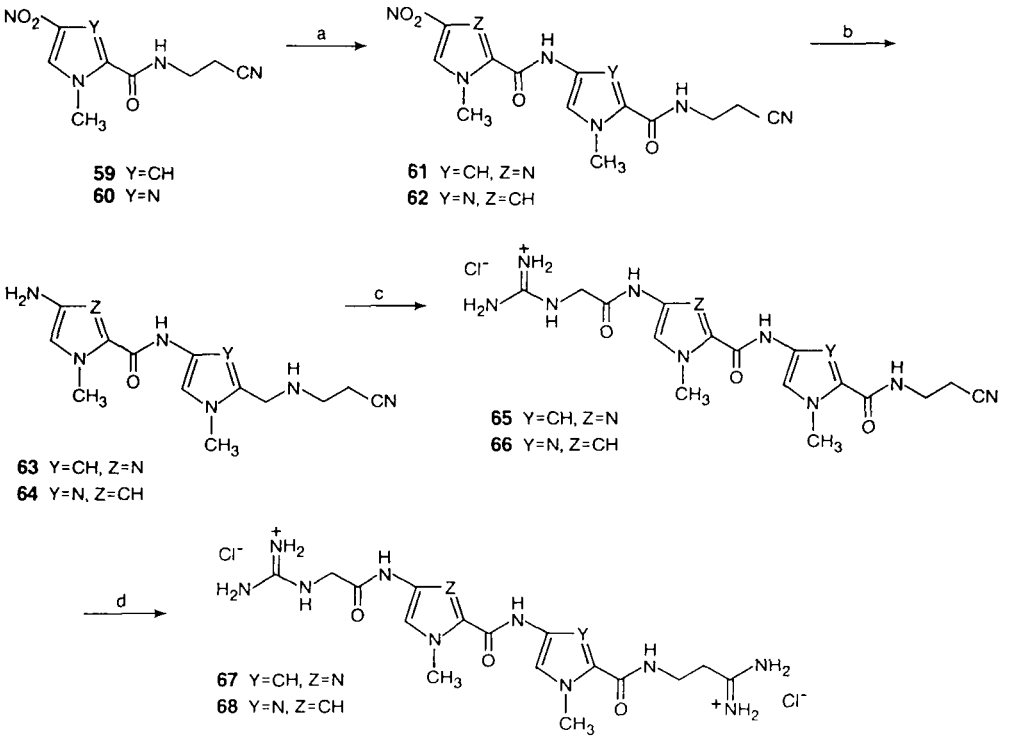
Formation of the amidine moiety was effected by a modification of the Pinner reaction.[29] Our observations agree with those of Baksheev[71] that the first step of the reaction of the cyano group, i.e. formation of the iminoester with an alcohol in the presence of hydrogen chloride is completed in 90 min and that longer reaction times promote side reactions resulting in lower yields. The iminoester reacts readily with ammonia in ethanolic solution within 1 h at ambient temperature. The




**Scheme 10.** Reaction conditions: (a), HCl in MeOH, then $NH_3$; (b), $(H_2N)_2CNHCH_2CO_2H$, $Cl^-$ and DCC; (c), HCl in EtOH, then $NH_3$, $Na_2SO_4$.

conditions for both reactions i.e. Pinner and condensation with guanidineacetic acid were first optimized with the monoimidazole derivatives **44** and **45a** to give **53** and **54** respectively.

In the final step of the synthesis, involving compounds **50** and **52**, the latter were first allowed to react with guanidylacetic acid, since the products, **55** and **56**




**Scheme 11.** Reaction conditions: (a), 5% Pd/C, $H_2$, then acyl chloride of **43** or acyl chloride or 1-methyl-4-nitropyrrole-2-carboxylic acid; (b), $H_2$, 10% Pd/C; (c), $(H_2N)_2CNHCH_2CO_2H$, $Cl^-$ and DCC; (d), HCl in EtOH, then $NH_3$.

respectively, could be readily purified. Subsequent introduction of the amidine moiety to afford the final target molecules **57** and **58** proceeded almost quantitatively (Scheme 10). The latter products were isolated initially as the hydrochloride salts contaminated with a little ammonium chloride and were quite soluble in protic solvents. Exchange of the counterion with sodium sulfate afforded the corresponding sulfate salts which were more readily purified by recrystallization. The composition of these polar compounds was established by FAB-MS.[45] In the case of the chloride salts $(M\text{-}Cl)^+$ or $(M\text{-}HCl\text{-}Cl)^+$ ions were observed for one or two salt functional groups respectively. For the corresponding sulfates, both $MH^+$ and $(M\text{-}HSO_4)^+$ ions were observed.

Synthesis of Oligopeptides Containing Both Pyrrole and Imidazole Moieties

The isomeric imidazole-pyrrole and pyrrole-imidazole dipeptides **61** and **62** were synthesized following similar procedures from **59** and **60** and 1-methyl-4-nitroimidazole-2-carboxylic acid and 1-methyl-4-nitropyrrole-2-carboxylic acid respectively (Scheme 11). After the reduction of the nitro groups the resulting amino compounds **63** and **64** were condensed with guanidineacetic acid hydrochloride in the presence of DCC to give **65** and **66** respectively. The Pinner reaction conditions on the latter compounds produced the target compounds **67** and **68**.

Sequence Specificity of Prototype Lexitropsins

These compounds bind to duplex DNA with constants in the range 1.06-1.98 $\times$ $10^6$ $M^{-1}$ but not to single stranded DNA. Since they bind to T4 DNA, it is inferred that, like the parent antibiotic netropsin, they are also minor groove selective.[72] This series of compounds exhibits a progressively decreasing preference for AT sites in binding studies with both native DNAs and synthetic oligonucleotides, and a corresponding increasing acceptance of GC base pairs as successive pyrrole units are replaced by imidazole groups. Footprinting experiments utilizing a 139 b.p. Hind III/NciI restriction fragment from pBR322 DNA revealed that these

lexitropsins, or information-reading oligopeptides, recognize more sites than the parent netropsin.[72] In addition some regions of enhanced nuclease action as the result of drug binding to the fragment were identified. The bis-imidazole compound in particular recognizes GC rich sites implying the formation of new hydrogen bonds between G-C(2)$NH_2$ in the minor groove to the additional $N_3$ imidazole nitrogens. It is clear however that, since the lexitropsins appear to tolerate the original $(AT)_4$ site, an N-methylimidazole group on the ligand will permit either a GC or AT base pair in the binding sequence. Another factor which appeared to be significant in molecular recognition is the high negative electrostatic potential of A.T regions of the minor groove which is likely to strongly influence binding of these bis-cationic species to DNA. This issue was addressed in the second generation lexitropsins.



## VI. SYNTHESIS OF MONOCATIONIC IMIDAZOLE LEXITROPSINS THAT DISPLAY ENHANCED GC SEQUENCE DEPENDENT DNA BINDING

The minor and major grooves of DNA have distinct electrostatic properties depending on the base composition. The deepest negative potential well is located in the minor groove in A.T rich runs.[73] Therefore dicationic lexitropsins or other ligands will have a bias for A.T rich sites regardless of the sequence specificity imposed by hydrogen bonding. This accounts for the "memory" that dicationic imidazole-lexitropsins display for the preferred site 5'-AATT of the parent netropsin. Accordingly, the guanidiniumacetyl moiety at the N-terminus of netropsin analogs was replaced by an N-formyl group giving monocationic lexitropsins.[28,62] The required compound **14** was already reported. The other compounds **69**, **70** and **71** were obtained by methods based essentially on the synthesis of **14** starting with the corresponding amino derivatives and using N-formylimidazole as formylating agent. However, owing to low solubilities of the amino derivatives containing imidazole moieties in methanol, DMSO was used as

solvent. Compound **69** was obtained in 49% yield, compound **70** in 51% yield and **71** in 81% yield.

DNase I footprinting on a 139 base pair Hind III/Nci I restriction fragment of pBR322 showed that the monocationic compound bearing two N-methylimidazole residues was found to exhibit dramatically altered specificity when compared with netropsin and preferred to bind to the unique sequence 5'-CCGT-3 (Figure 3)'.[74] Thus not only is the imidazole moiety recognizing GC sites but, to a degree, is reading DNA sequence. The ability of the compound to discriminate between

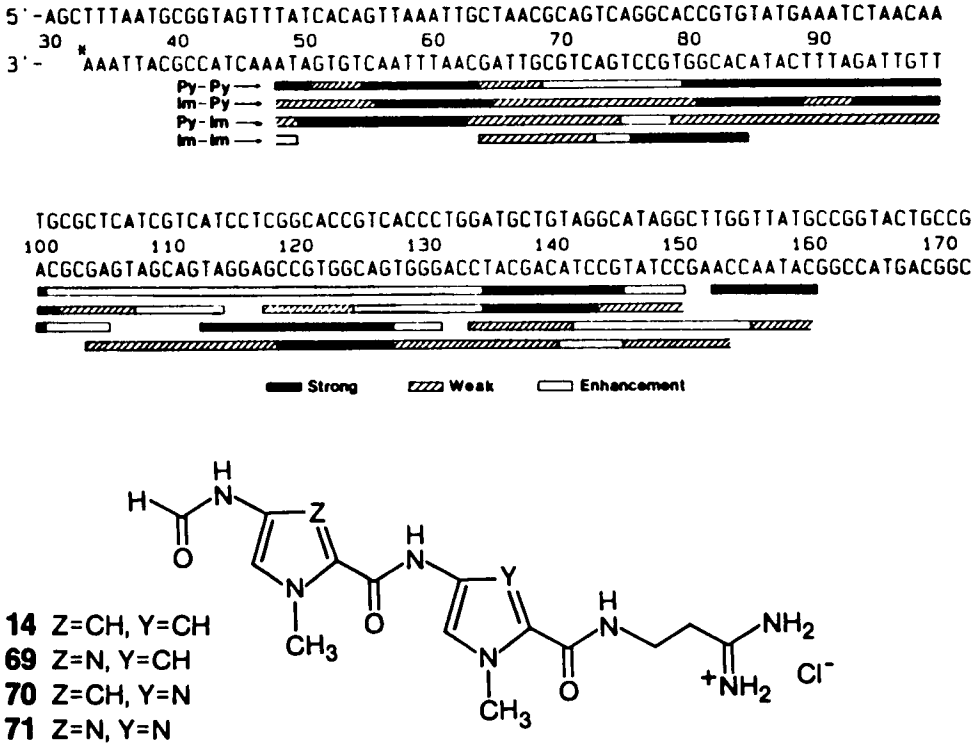






**Figure 3.** Sequence specific DNA binding of second generation lexitropsins from DNase I footprinting on a Hind III/Nci I restriction fragment of pBR322.

the preferred sequence and other possible sequences preseng e.g. (all 5'-3') TGCC, TGCG, AGCG, AGGG, and AGCC appears related to structural differences between these sequences which are sensed by the lexitropsin. Comparison of the

results of this study with those involving the prototype series of lexitropsins[72] suggests that the reduction of charge and/or alteration of one of the van der Waals contacts between the ligand and DNA has a significant effect on binding specificity.

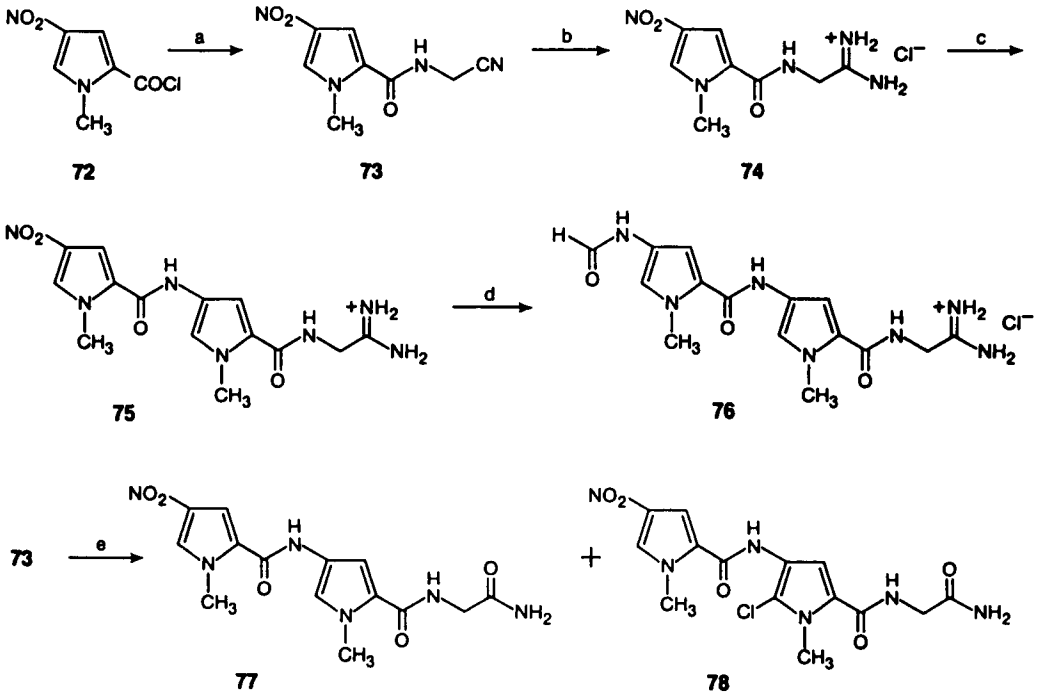
## VII. SYNTHESIS OF TRUNCATED 3'-TERMINAL GC READING LEXITROPSINS

The ligand formyl-Im-Im-$CH_2CH_2$-Am$^+$ is shown by DNase I footprinting on the Hind III/Nci I fragment from the pBR322 DNA to be specific for the sequence 5'-CCGT indicating that the AT bias of netropsin has been overcome.[74] Independent $^1$H-NMR studies of this lexitropsin when challenged with the sequence d-[CATGGCCATG]$_2$ indicate that it binds preferentially to the 5'-CCAT sequence.[75] Therefore, replacement of the pyrrole group(s) with hydrogen bond accepting heterocycles, such as imidazole, evidently affords compounds that can recognize G.C sites by hydrogen bond formation to G-(C2)-$NH_2$.[18,72]

It was also deduced from the latter NMR study that the reading of a 3'-terminal A.T base pair is presumably dictated by the van der Waals interactions between the methylenes at the C-terminus of the lexitropsin and the DNA. These methylene groups would enter into steric contact with the guanine-$NH_2$ moiety of a G.C base pair, thereby preventing the binding of the C-terminus of netropsin analogues to a G.C site and forcing the recognition of an A.T pair by default. In order to test this interpretation and to examine the influence of the methylenes at the C-terminus in the molecular recognition of netropsin analogs to DNA, we have designed a "truncated" lexitropsin **76** (with only one methylene group at the C-terminus) for this study. This investigation is supported by comparison with the $^1$H-NMR analysis of lexitropsin **14**,[28] which has the "normal" ethylene group at the C-terminus.

The initial approach to the synthesis of **76** based on literature methods[76] proved to be unsatisfactory owing to the quite different chemical properties of amidoacetonitrile compared with amidopropionitrile present in the natural antibiotics distamycin and netropsin. Arcamone et al. found the nitro group in **73** to

be resistant to hydrogenation and employed an unusually large proportion of Pd/C catalyst (1:2 catalyst:compound) and a long reaction time (4 h) to afford a modest yield (53%) of the product of coupling with the acyl chloride **72**. We avoided the inconvenience of poisoning of the catalyst by the amidoacetonitrile in **73** by first converting the nitrile group to an amidine moiety in **74** by a Pinner reaction (see Scheme 12).[62] The nitro group in **74** was then readily reduced catalytically and




**Scheme 12.** Reaction conditions: (a), $H_2NCH_2CN$, $iPr_2EtN$; (b), HCl in MeOH, then $NH_3$ in MeOH; (c), $H_2$, Pd/C then add **72**; (d), $H_2$, Pd/C then $HCO\text{-}O\text{-}COCH_3$; (e), $SnCl_2$, then NaOH, then add **72**.

coupled with the acyl chloride **72** to give the dipeptide **75**. Catalytic reduction of the nitro group and reaction with acetic formic anhydride afforded the desired final product **76** in 62% yield.[77] It may be noted that the alternative use of stannous chloride for reduction prior to the coupling step affords the amide derivatives **77** and **78** (Scheme 12).

The binding specificity and dynamic properties of the truncated lexitropsin **76** which has one methylene group at the C-terminus, and the "normal" lexitropsin **14** which has two methylene groups at the C-terminus to the decamer d-[CGCAATTGCG]$_2$ deduced by high field $^1$H-NMR.[78] Selective chemical shift changes of AH8(5), TH6(6,7) and GH8(8) and NOE evidence locate the truncated lexitropsin on the ATTG sequence. In contrast the comparable chemical shift and NOE data on the normal lexitropsin locate this ligand at the AATT site on the DNA. These results clearly demonstrate that the van der Waals interactions between the



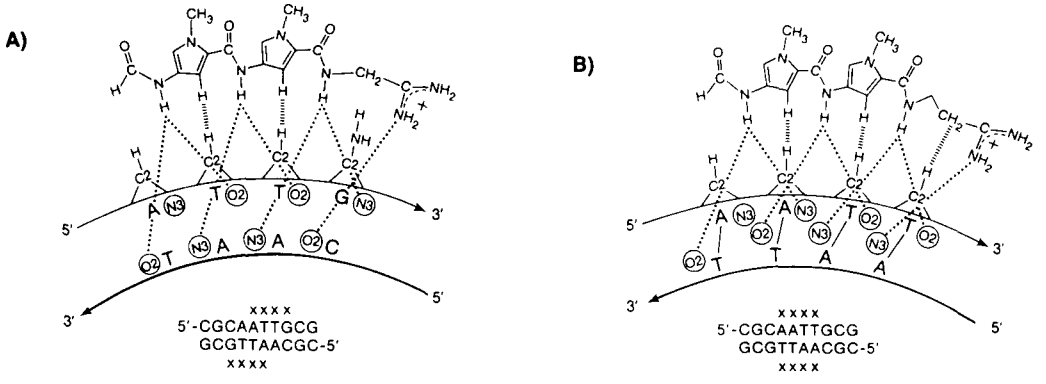



**Figure 4.** DNA sequence specific molecular recognition deduced from high field $^1$H-NMR studies for (A) truncated lexitropsin permitting recognition of 3'-GC and (B) normal 3'-AT reading lexitropsin.

methylene group at the C-terminus of lexitropsins similar to **14**, and what would be the guanine-2-$NH_2$ group, are responsible for reading the 3'-terminal AT base pair (Fig. 4). The rate of exchange of **5** between the two equivalent ATTG sites is ≈185 $s^{-1}$ at 294°K with $\Delta G^{\ddagger} \approx 58 \pm 5$ kJ $mol^{-1}$, whereas the exchange of **8** between the two equivalent AATT sites is faster (>180 $s^{-1}$) even at 277°K.[78]

## VIII. SYNTHESIS OF PROTOTYPE SEQUENCE SPECIFIC DNA EFFECTORS: MINOR GROOVE ALKYLATORS

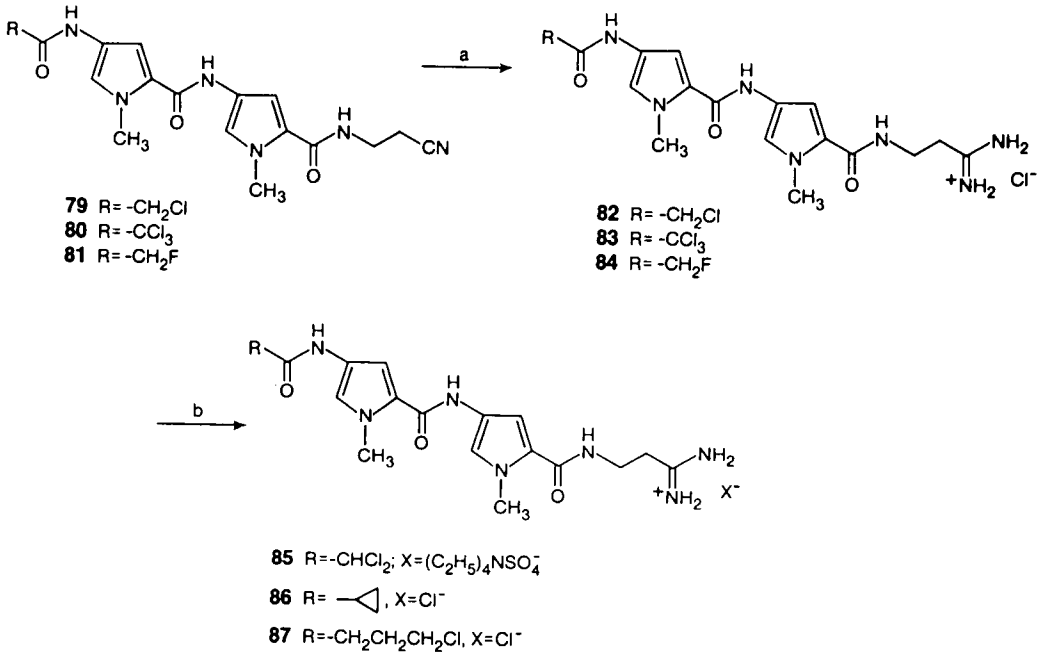
The ultimate synthetic goal of this research is to develop sequence specific DNA effectors based on the lexitropsin oligopeptide concept. To this end we next explored the synthesis of oligopeptides bearing alkylating moieties. Natural precedents exist for minor groove alkylators in the pyrrolo(1,4)benzodiazepine antibiotics[79] and in CC-1065.[80] Therefore oligopeptide agents bearing potential alkylating functions were prepared to be analogous with the former, and examples bearing activated cyclopropyl groups were designed to be analogous to the latter.



The new oligopeptide agents carry potential alkylating moieties viz. mono, di, and trichloroacetyl, fluoroacetyl, or cyclopropylcarbonyl. All these groups were introduced by allowing the corresponding acyl chlorides to react with 1-methyl-4-[(1-methyl-4-amino-pyrrole-2-carboxamido)pyrrole-2-carboxamido]propionitrile **10** which had been used in our total synthesis of netropsin **1**.[28] The monochloroacetyl derivative **79** obtained by this procedure had been employed in the synthesis of oligopeptides with trimethylammonium and triethylammonium moieties as alternative charged end groups to that of the guanidineacetyl in the antibiotic netropsin.[81]

After introduction of an acyl group into the oligopeptide the amidine moiety is normally generated via the Pinner procedure[29] by reacting with ethanol in the presence of hydrogen chloride and subsequent ammonolysis.[82] However, in case of the dichloroacetyl **85** and cyclopropylcarbonyl **86** derivatives (Scheme 13), better results were obtained by direct reaction of the corresponding acyl chlorides

with a compound already containing an amidine moiety i.e. 1-methyl-4-[(1-methyl-4-aminopyrrole-2-carboxamido)pyrrole-2-carboxamido]propionamidine hydrochloride **13**. In condensation of acyl chlorides with amino oligopeptides, Hunig's base ($NEtiPr_2$) or N-ethylmorpholine were used as bases. N-Ethylmorpholine is especially appropriate in reactions of acyl chlorides with amino oligopeptides containing amidine groups, because the N-ethylmorpholine hydrochloride formed is readily soluble in acetonitrile and hydrochloride salts of acylamino-oligopeptide-amidines formed in the condensation process are




**Scheme 13.** Reaction conditions: (a), HCl in EtOH, then $NH_3$; (b), RCOCl and $Et(iPr)_2N$.

insoluble in the same solvent and can be readily isolated in the pure state. The dichloroacetyl derivative was isolated as the tetraethylammonium sulfate. This procedure permitted the elimination of ammonium chloride contaminating the product.

Introduction of an alkylating moiety in place of the charged guanidinium-acetyl end group in netropsin proved to be effective in increasing the cytostatic activity against certain human tumor cell lines by a factor of up to 45 compared with the parent natural product netropsin.[82] Especially noteworthy is the chloroacetyl derivative **82** which was 15 to 45 times more potent than netropsin **1** and 3 to 18 times more potent than distamycin **2** against all murine and human tumor cell lines examined with the exception of Molt/4F against which it equaled distamycin in activity. It is evident that an active alkylating group is essential for cytostatic activity against both L1210 and P388 leukemias, i.e. it is insufficient solely to bind to DNA as exemplified by control compound **87** which contains an insulating methylene chain between the halogen and the acyl group. Similarly, the lower alkylating property of the fluoro group is reflected in the relative activities of **82** and **84**. It should be noted, however, that the ability of this class of agents to bind to DNA[72,74,75] contributes to their cytostatic activity, as attested by the activity of **84** against Molt/5F, Raji and Namalva which is comparable with that of distamycin. Similarly the non-alkylating compound **87** shows modest activity against the murine mammary carcinoma PM3A cell line.

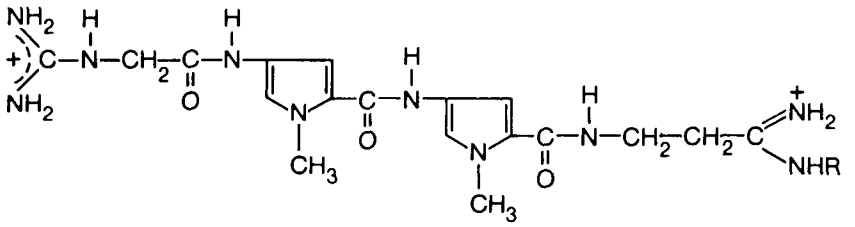
Compound **86** which contains an activated cyclopropane group was modeled on the potent minor groove DNA binding agent CC-1065. This agent has been shown to bind selectively to $(A.T)_4$ sequences, like netropsin, but forms a covalent bond via the activated cyclopropane moiety.[80] In fact compound **86** selectively inhibits Molt/4F at a fourfold lower concentration than distamycin. The other agents tested **83**, **84** and **85** had cytostatic activity comparable with that of distamycin.

## IX. SYNTHESIS OF SPIN-LABELED NETROPSIN AS A PROBE FOR CELLULAR UPTAKE AND SUBCELLULAR DISTRIBUTION

As was stated at the outset, while oligonucleotide based agents meet some of the criteria for viable antisense probes i.e. high binding specificity, and resistance to intracellular nucleases in the case of β-oligomer methyl phosphonates and α-oligomers, they often suffer the serious disadvantage of difficulty of penetration of the cellular membrane. In order to assess the cellular uptake and subcellular distribution a nitroxide spin-labeled netropsin was prepared and studied in cell cultures.[83]

The oligopeptide nitrile was treated with dry HCl in ethanol. Subsequent treatment of the iminoether with 4-amino-2,2,6,6-tetramethylpiperidinyl-N-oxide afforded the spin-labeled netropsin **88** in 69% yield.[83]





**1** Netropsin R=H

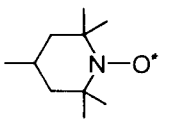
**88** SL-net R= 

**Figure 5.** Structure of nitroxide spin-labeled netropsin used for subcellular distribution studies.

The nitroxide spin-labeled netropsin **88** (Figure 5) was studied by EPR spectroscopy with respect to its uptake and localization in living KB cells. Whereas the drug was taken up readily, there was no detectable binding to the membrane, relatively little drug in the cytoplasm, but significant concentration of the drug in the

cell nucleus. In contrast a control nitroxide, which does not bear a DNA interactive group, diffuses to all parts of the cell equally.[84] There is no evidence that the drug binds to other cellular macromolecules, e.g. a carrier protein. The results also show that the drug, whether in the cell nucleus or the cytoplasm, is relatively long-lived and therefore apparently not subject to rapid intracellular degradation. The EPR signal in the latter site corresponds to a relatively freely rotating radical. The drug exhibited good intracellular stability up to 25 h. While a $\Delta$Tm of 24° between the spin-labeled netropsin and calf thymus DNA confirms strong binding, the absence of any DNA elongation by viscometry is consistent with non-intercalative exterior binding which is confirmed to be minor groove specific by binding of the agent to T4 DNA with a $\Delta$Tm of 17.5°C. The sequence specificity of the DNA binding of the spin-labeled drug was confirmed by MPE footprinting on calf thymus DNA to be very similar to that of the parent netropsin, i.e. selective for AT-rich sites, with minor differences of protection afforded by introduction of the nitroxide label.



These results represent the first direct evidence, of which we are aware, of preferential minor groove binding of such oligopeptide antitumor antibiotics to the nucleus of living tumor cells. The results also demonstrate that lexitropsins meet many of the criteria for viable cell regulatory probes, i.e. relative ease of access into the cell, preferential binding in the cell nucleus and acceptable intracellular stability, in contrast to oligonucleotide-based probes, and sequence recognizing capabilities.

## X. CONCLUSIONS AND PROSPECTS

The combination of synthetic organic chemistry with DNA footprinting methodology is a powerful way to uncover the structural elements which determine ligand-DNA sequence recognition. The lexitropsin concept and model has clarified the importance of hydrogen bond accepting and donating sites in the component heterocycles, of the number and placement of cationic changes and the effects of

individual van der Waals contacts in determining sequence specific binding, mechanism and rate of ligand-DNA exchange. The novel oligopeptide lexitropsins that have been developed to date exhibit many useful properties. They have good cellular uptake characteristics, acceptable intracellular stability, concentrate in the cell nucleus and form stable complexes by binding in a sequence specific manner to the minor groove of double helical DNA.

However several synthetic challenges remain. Sensitivity to cellular peptidases is anticipated in lexitropsins bearing individual base-pair recognizing α-amino acids. The longer lexitropsins necessary to locate and bind to a unique cellular control sequence (~15 base pairs for the human genome) pose problems with respect to solubility and phasing. The latter problem arises owing to the lack of precise dimensional correspondence between oligopeptides and their oligonucleotide counterparts. The incorporation of alternative heterocyclic components (e.g. oxazole, thiazole, triazoles), poses certain synthetic problems since, surprisingly, much of the required heterocyclic chemistry is unavailable. The lexitropsin concept is readily applicable to other groove binding models susceptible to appropriate structural modification. These and related synthetic challenges await the ingenuity of the organic chemist.



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