Distamycin-NA: A DNA Analog with an Aromatic Heterocyclic Polyamide Backbone

Part 2

Solid-Phase Synthesis of Distamycin-NAs Containing the Nucleobase Uracil: Unexpected Solvent Participation in the Coupling Step

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The synthesis of the Fmoc-protected amino acid **2** is presented. First attempts of amide-bond formation to the homodimer **4** in solution showed only poor coupling yields indicative for the low reactivity of the amino and carboxy groups in the building blocks **1** and **2**, respectively (*Scheme 1*). Best coupling yields were found using dicyclohexylcarbodiimide (DCC) without any additive. The oligomerization of building block **2** adopting the Fmoc ((9*H*-fluoren-9-ylmethoxy)carbonyl) solid-phase synthesis yielded a mixture of N-terminal-modified distamycin-NA derivatives. By combined HPLC and MALDI-TOF-MS analysis, the N-terminal functional groups could be identified as acetamide and *N*,*N*-dimethylformamidine functions, arising from coupling of the N-terminus of the growing chain with residual AcOH or DCC-activated solvent DMF. An improved preparation of building block **2** and coupling protocol led to the prevention of the N-terminal acetylation. However, 'amidination' could not be circumvented. A thus isolated tetramer of **2**, containing a lysine unit at the C-terminus and a *N*,*N*-dimethylformamidine-modified N-terminus, not unexpectedly, showed no complementary base pairing to DNA and RNA, as determined by standard UV-melting-curve analysis.

1. Introduction. – In an effort to discover DNA analogs containing conceptually new backbone surrogates, we recently disclosed the design and synthesis of the monomeric building block of distamycin-NA [2]. In the design of distamycin-NA, we wished to combine the elements of structural preorganization of the DNA minor groove binders distamycin and netropsin (crescent molecular shape) with the known complementary base-base recognition elements of the nucleic acids. The repetitive monomer unit is built from a thiophene-core unit connected *via* a biaryl-like axis to the DNA recognition unit, the nucleobase uracil. Next to the biaryl-like axis on the thiophene heterocycle are located a carboxy and an amino substituent allowing for oligomerization *via* peptide coupling. The conformations of the repetitive unit of distamycin-NA can be described by the three torsion angles α, β, and γ (see **B** in *Fig. 1*; compare with **A** (distamycin) and **C** (part of B-DNA)).

The monomer **1** was obtained in a five-step synthesis starting from commercially available precursors. Structural analysis of methyl 4-nitro-3-(1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)thiophene-2-carboxylate, a close relative of **1** in which the NH_2 group is replaced by an NO_2 group, clearly showed that the plane of the uracil unit is

[1]) Taken in part from the Ph.D. thesis of *G.S.* [1].

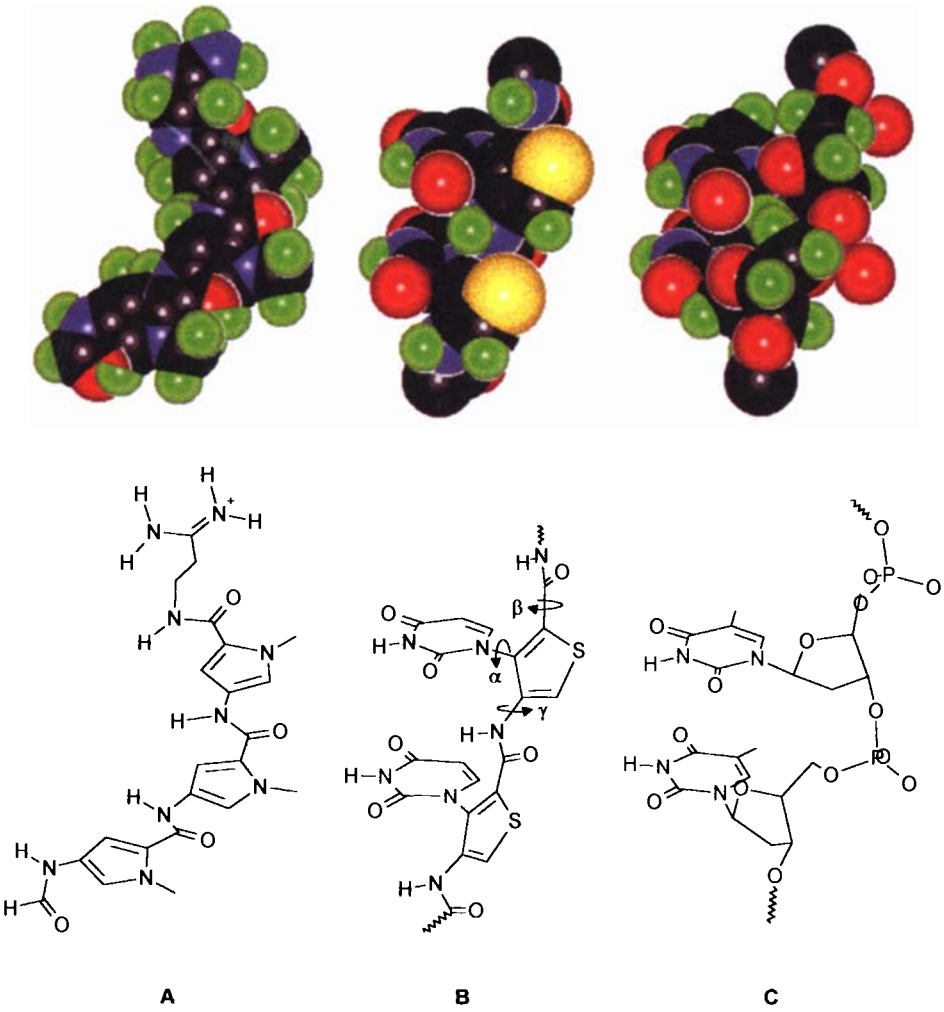
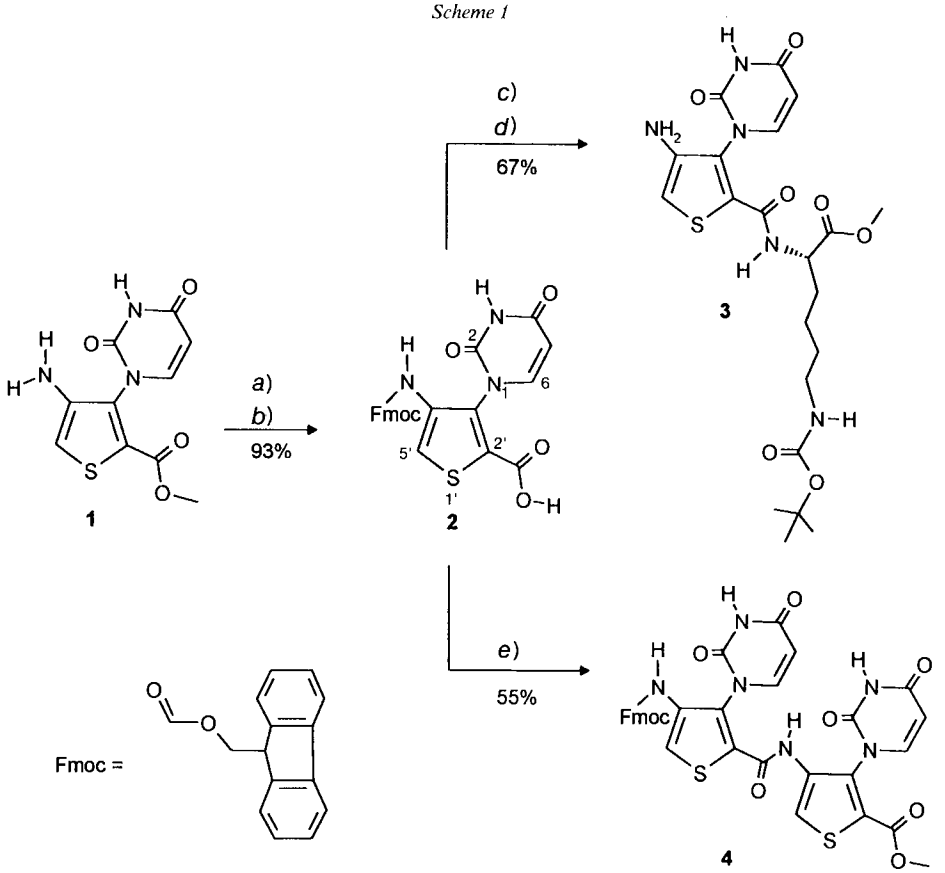


Fig. 1. *Conformations of distamycin* (**A**), *part of distamycin-NA* (**B**), *and part of B-DNA* (**C**). *Top*: space-filling representation; *bottom*: stick representation.

rotated, as expected, by *ca.* 90° relative to that of the thiophene unit. To determine the rotational barrier around the biaryl-like axis, variable-temperature NMR experiments were performed on the corresponding dimer. From these experiments, a barrier of 19 ± 1 kcal/mol was calculated. This value is in a range allowing, nevertheless, efficient interconversion of rotamers at room temperature and slightly elevated temperatures. Therefore, distamycin-NA is a dynamic atropisomeric system. These initial results stimulated us to synthesize oligomers. Here we present first results on the oligomerization of the protected amino-acid building block **2** adopting the general scheme of Fmoc ((9*H*-fluoren-9-ylmethoxy)carbonyl) solid-phase peptide synthesis.

2. Results and Discussion. – 2.1. *Fmoc-Protected Amino-Acid Building Block* **2** *and Preliminary Coupling Reactions in Solution.* The ester function of methyl 4-amino-3-(1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)thiophene-2-carboxylate (**1**) was first saponified with KOH in dioxane/H_2O 4:1. After adjusting the pH of the solution to 9–10 with sat. $NaHCO_3$ solution, acylation of the amino-acid intermediate was effected *in situ* by addition of an excess of Fmoc-Cl in small portions which afforded **2** in 93%[2]) yield (*Scheme 1*).

Scheme 1



a) KOH, dioxane/H_2O 4:1, 10 min. *b*) Fmoc-Cl, dioxane/H_2O 1:1, 1.3 h. *c*) H-Lys(ε-Boc)-OMe · HCl, DCC/BtOH/Et_3N, DMF, 1.5 h. *d*) Piperidine, dioxane, 20 min. *e*) **1**, DCC, DMF, 6 h.

[2]) The excess of the reagent Fmoc-Cl led to decomposition products which were removed by column chromatography (silica gel). However, due to the high polarity of **2**, initially AcOH was used in the mobile phase. The isolated product **2**, therefore, reproducibly contained 3–7% (^{1}H-NMR) of AcOH as an impurity which could not be removed, even by repetitive coevaporation with toluene and long drying *in vacuo*. In later preparations, column chromatography could be replaced by precipitation of **2**, thus allowing its isolation in analytically pure form.

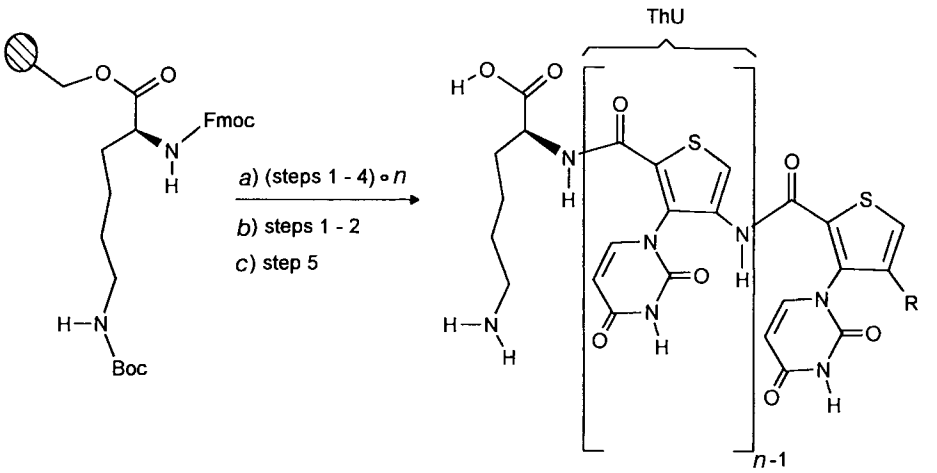
Preliminary experiments to amide-bond formation followed by a Fmoc-deprotection step were performed in solution using building block **2** as the carboxy component and ε-Boc-protected lysine methyl ester (Boc = (*tert*-butoxy)carbonyl) as the amino component. Using conventional DCC/BtOH (dicyclohexylcarbodiimide/1-hydroxy-1*H*-benzotriazole) coupling [3] and Fmoc-deprotection chemistry [4], the lysine derivative **3** could be isolated in 67% yield. For the synthesis of the homodimer **4**, several preliminary attempts using the coupling reagents as, *e.g.*, TBTU (*O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate) [5] or BOP ((1*H*-benzotriazol-1-yloxy)-tris(dimethylamino)phosphonium hexafluoroborate) [6] led to no amide-bond formation at all. Therefore, comparative coupling reactions based on DCC in DMF with different additives were tested in solution [7]: DCC with no additive, DCC/BtOH, DCC/BtoOH (3-hydroxy-1,2,3-benzotriazin-4(3*H*)-one). The coupling by DCC without any additive worked best affording the dimer **4** in up to 55% yield. Addition of BtOH and BtoOH had a decelerating effect on the coupling speed. These results pointed out that in absence of any additive, the *in situ* prepared symmetric anhydride of **2** was more reactive than the BtO- and BtoO-activated esters in this system. Neither the addition of DMAP (4-(dimethylamino)pyridine) nor 1-methyl-1*H*-imidazole [8] caused an improvement on the efficiency of the coupling reaction. On the contrary, substantial amounts of by-products in which the Fmoc group was removed were formed. The encountered difficulties in the coupling reactions are indicative for the weak nucleophilicity of the aromatic and sterically hindered amino function in **1**.

2.2. *Distamycin-NA Hexamer on Solid Support.* The most efficient and economic way to produce oligomers, taking into account the previously described moderate coupling yields in solution, seemed to be the solid-phase technique in which excess of reagents and the phase characteristics of the solid support might overcome the difficulties in the coupling steps. Between the two widely used protecting-group strategies, Boc and Fmoc, we chose the latter technique because of the milder deprotection and detachment steps. For the synthesis of oligomers **5**–**8** the commercially available *Wang* resin carrying the Fmoc/ε-Boc-protected lysine as the starter unit which is connected to the solid support *via* an ester-bond linkage was used (*Scheme 2*; *Fig. 2*). Lysine was chosen as the starter unit, in analogy to *Buchardt*, *Nielsen*, and coworkers [9], to increase the water solubility of the oligomer. Furthermore, the introduced positive charge might enhance DNA binding. Oligopeptide synthesis was performed manually in analogy to standard protocols in peptide synthesis as described by *Atherton* and *Sheppard* [10]. The symmetric *in situ* formed anhydride of **2**, generated by treatment with DCC in DMF, was used as the activated building block.

The synthesis of a hexamer of **2** was attempted using the reaction cycle depicted in *Scheme 2*. Coupling efficiencies per step were analyzed by a UV-based Fmoc assay[3]). From this assay it was found that building block **2** coupled in 95% to the solid-phase-bound lysine within 3 h. The second coupling step, involving the first amide-bond formation between two units of **2** again proceeded with a yield of 96%. However, the coupling efficiency decreased substantially (to 68%) in the third coupling step. Finally, after six

[3]) The ninhydrin ('*Kaiser*') color test [10] turned out to be useless because no significant staining after *N*-deprotection of the growing polyamide was observed. The yield after each coupling step was, therefore, determined by UV spectroscopy at the 267-nm absorbance of aliquots of the Fmoc-deprotection solutions.

Scheme 2



	Step	Reagent/Solvent	Volume [ml]	Time	Repeat
Chain assembly	1) deprotection	20% piperidine/DMF	0.5	3 min	1
		"	0.5	7 min	1
	2) wash	DMF	0.5	1 min	10
	3) coupling	Soln. *A* or *B* (see *Exper. Part*)	0.5	3-23 h	1-2
	4) wash	DMF	0.5	1 min	10
Detachment/ deprotection	5) hydrolysis	95% CF_3COOH 5% H_2O	1	2 h	1
		"	0.5	1 min	3

couplings, only 25% of the initially observed amount of dibenzofulvene derivatives (from Fmoc) were detected. Deprotection and detachment using CF_3COOH/H_2O 95:5 produced the crude mixture of oligomers that were further analyzed.

2.3. *Analysis of the Released Polyamide Mixture.* The analysis of the crude synthesis mixture was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Screening for suitable matrix compounds (2,5-dihydroxybenzoic acid, salicylic acid (= 2-hydroxybenzoic acid), and sinapic acid = 3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid) was first carried out. Sinapic acid worked best for the range of the higher masses ($m/z > 1000$), whereas 2,5-dihydroxybenzoic acid yielded better results in the mass range below ($m/z < 1000$). The MS analysis with salicylic acid as matrix was unsatisfactory. In the positive mode, more ions than in the negative mode were detected. The spectrum of the crude reaction mixture under optimized matrix conditions is shown in *Fig. 3*. The spectrum shows six sets of multiple peaks in which the accurate masses of the distamycin-NAs **7a**, **8a**, **9a**, and **10a** could be

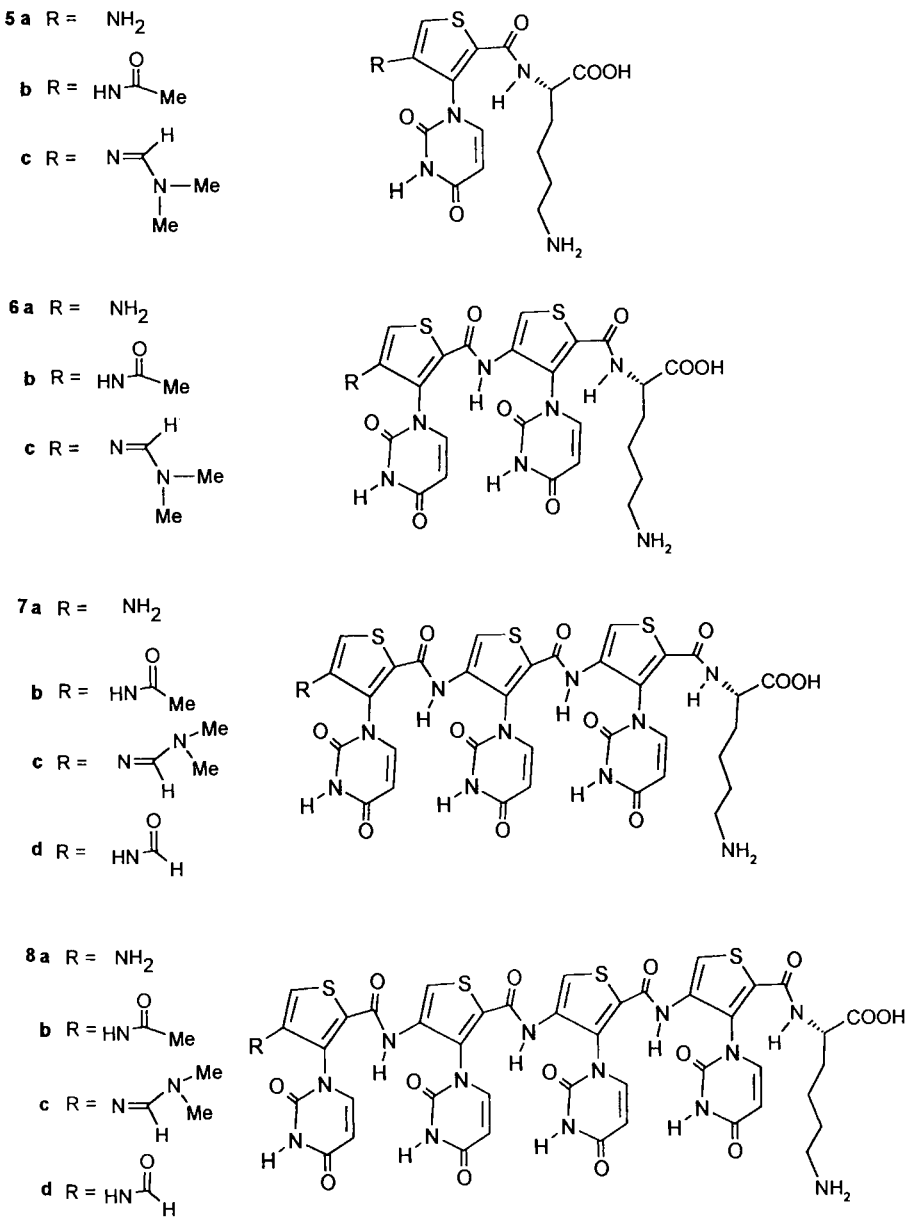


Fig. 2. *Oligomers prepared as shown in* Scheme 2

identified. Besides the desired polyamides, also mass signals of compounds showing increased masses by + 43 and + 56 dalton were observed, in addition to their ions of alkali-metal adducts such as $[M + Na]^+$ and $[M + K]^+$ [4]).

[4]) Throughout the text, we use the fragment masses + 43 and + 56. Please note that the actual measured mass difference is, therefore, lower by one unit each, due to replacement of the fragment by a H-atom (see *Table 1*).

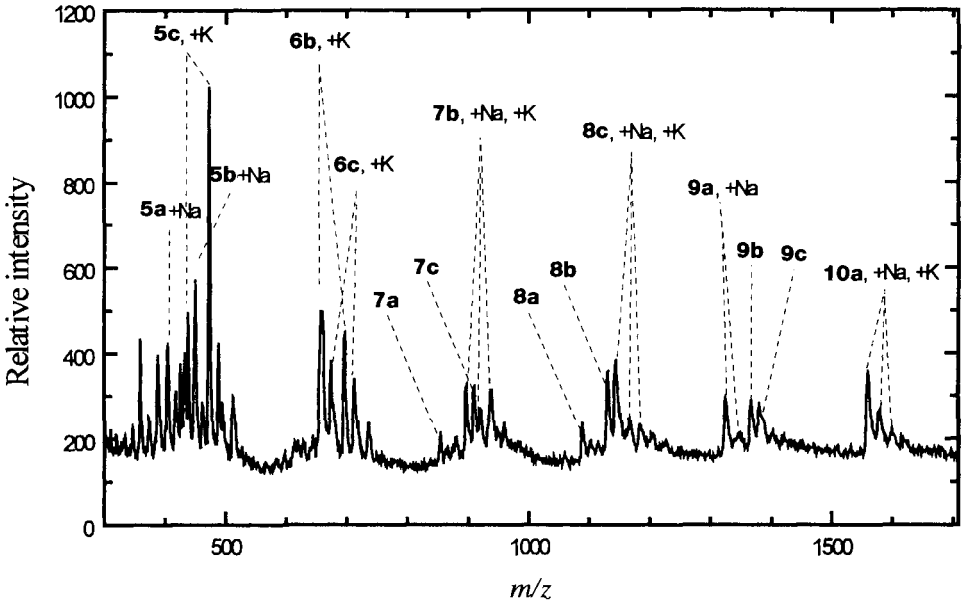


Fig. 3. *MALDI-TOF Mass spectrum of the crude mixture after detachment from solid support* (matrix: sinapic acid, average of 41 individual laser shots)

Separation of the crude mixture into pure components was achieved by reversed-phase HPLC (*Fig. 4*). The HPLC of the crude mixtures shows, besides the first eluted peak, three sets of two well-resolved peaks in regular intervals in decreasing intensity. To correlate the HPLC and MS, each fraction was collected and again analyzed by MALDI-TOF-MS. *Table 1* contains the complete correlation between found masses and collected HPLC fractions.

It was found that only peaks with minor intensity corresponded to the distamycin-NAs **6a**, **7a**, and **8a** with an unmodified terminal amino function. The + 43 adducts led to significantly more apolar polyamide derivatives, while the + 56 adducts were more polar than the parent distamycin-NAs. To exclude that the measured additional masses of + 43 and + 56 arise from combinations of alkali-metal adducts as, *e.g.*, $[M - H + Li + K]^+$, NaCl or KCl were separately added to a representative probe of **7b**. The resulting spectra of **7b** are depicted in *Figs. 5* and *6*. Since, in both cases, the same lowest *m/z* peak of 894 ± 1 was observed, one can safely assign this mass to a covalently modified derivative of **7a** with an enhanced mass of 43 dalton. The regular mass intervals of + 22 in *Fig. 5* can be attributed to the Na^+ adducts of $[M + Na]^+$, $[M - H + 2Na]^+$, and $[M - 2H + 3Na]^+$ *etc.* *Fig. 6* represents the corresponding K^+ adducts $[M + K]^+$, $[M - 1H + 2K]^+$ and $[M - 2H + 3K]^+$ *etc.* obtained after addition of KCl. The same experiments were also performed with a probe of fraction **8c** (not shown) which clearly demonstrated the presence of a further covalent modification of distamycin-NAs with an enhanced mass of 56 dalton.

The amount of the mass differences gives rise to the hypothesis that derivation took place at the N-terminus. For the observed + 43 additive, it seemed reasonable to assume an N-terminal acetamide function as the structural element. The origin of it can be traced

Table 1. *Results of the Mass Analyses of the Collected HPLC Fractions.* For the abbreviation 'ThU', see *Scheme 2.*

No.	Compound	t_R [min]	Int. [%]	Mass calc.	Mass found
				$[M + 1]^+$, $[M + Na]^+$, $[M + K]^+$	$[M + 1]^+$, $[M + Na]^+$, $[M + K]^+$
5c	Me_2N-CH=N-(ThU)-Lys-COOH	8.3	19.0	437.41, 459.41, 475.51	437.3, 459.7, 475.6
b	AcNH-(ThU)-Lys-COOH	10.8	12.4	424.41, 446.41, 462.51	423.9, 446.0, 462.3
6c	Me_2N-CH=N-$(ThU)_2$-Lys-COOH	11.8	8.3	672.63, 694.63, 710.73	672.2, 694.5, 710.1
a	$NH_2(ThU)_2$-Lys-COOH	13.1	1.1	617.63, 639.63, 655.73	618.1, 640.3, 656.8
b	AcNH-$(ThU)_2$-Lys-COOH	18.0	11.4	659.63, 681.63, 697.73	660.0, 682.1 698.3
7c	Me_2N-CH=N-$(ThU)_3$-Lys-COOH	19.7	10.1	907.86, 929.86, 945.96	909.6, 931.1, 947.3
a	NH_2-$(ThU)_3$-Lys-COOH	22.1	2.0	852.86, 874.86, 890.96	853.7, 876.2, 891.1
b	AcNH-$(ThU)_3$-Lys-COOH	28.5	8.4	894.86, 916.86, 932.96	895.3, 916.5, 933.2
8c	Me_2N-CH=N-$(ThU)_4$-Lys-COOH	30.0	7.0	1143.08, 1165.08, 1181.18	1144.2, 1165.2, 1180.4
a	$NH_2(ThU)_4$-Lys-COOH	32.7	1.6	1088.08, 1110.08, 1126.18	1088.1, 1110.6, 1127.3
b	AcNH-$(ThU)_4$-Lys-COOH	39.2	4.2	1130.08, 1152.08, 1168.18	1131.1, 1153.5, 1167.2
9c	Me_2N-CH=N-$(ThU)_5$-Lys-COOH	39.9	2.0	1378.30, 1400.30, 1416.40	1378.3, 1400.3, 1416.8
b	AcNH-$(ThU)_5$-Lys-COOH	48.5	1.9	1365.30, 1387.30, 1403.40	1365.8, 1387.6, 1404.1
10a	NH_2-$(ThU)_6$-Lys-COOH	–	–	1558.53, 1580.53, 1596.63	1560.1, 1579.1, 1598.7

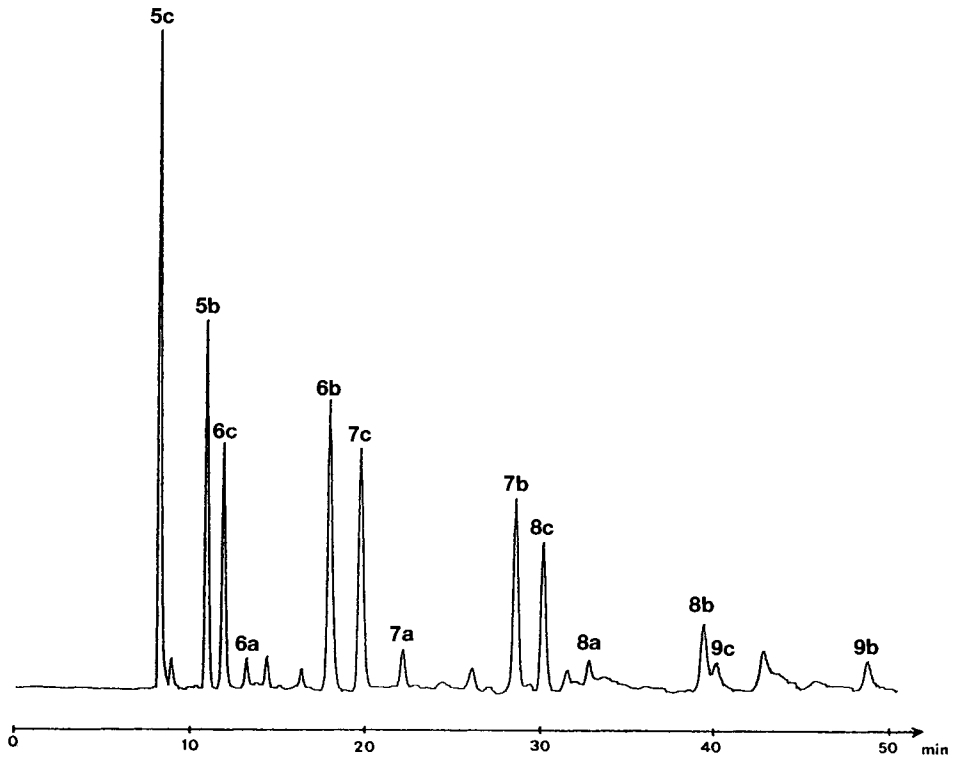


Fig. 4. *Crude mixture from solid-phase synthesis analyzed by HPLC. Nucleosil-CC-250/4-5-C18* column. (*Macherey & Nagel*); eluent *A*: 0.1% CF_3COOH in H_2O; eluent *B*: MeCN/H_2O 9:1; linear gradient: 9–20% *B* in 50 min; flow rate 1 ml/min; UV detection at 260 nm.

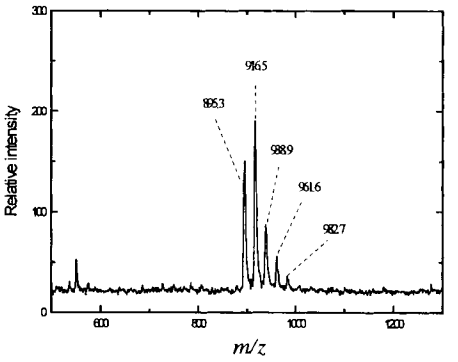


Fig. 5. *MALDI-TOF Mass spectrum of* **7b**, *with matrix 2,5-dihydroxybenzoic acid + 1% NaCl.* Average of 11 individual shots.

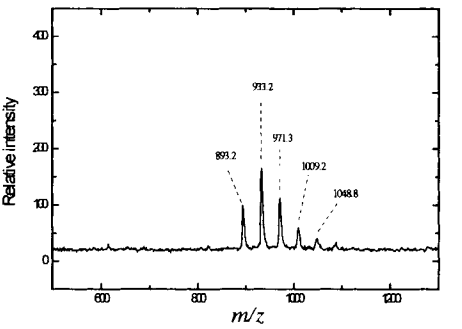


Fig. 6. *MALDI-TOF Mass spectrum of* **7b**, *with matrix 2,5-dihydroxybenzoic acid + 1% KCl.* Average of 11 individual shots.

back to AcOH being present as 3–7% (unremovable) by-product in the monomer building block **2**. In spite of the minor quantity, DCC-activated AcOH appeared in this solid-phase approach as a serious competitive coupling inhibitor. This serious competi-

tion was not noticed during the coupling reaction in solution. For the observed more polar + 56 additive, the hypothesis of an *N,N'*-dimethylformamidine function seemed obvious. It had already been suggested earlier by *Moffatt* and *Lerch* [11] that DMF in the presence of DCC can effectively 'amidinate' arylic amines (*Fig. 7*). Final proof for those hypotheses was obtained from ^{1}H-NMR analysis of the compounds in fractions **5b** and **5c**, which clearly showed the presence of the two substructural units mentioned [1].

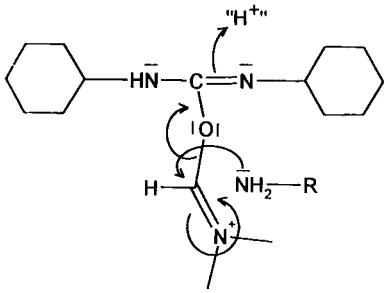


Fig. 7. *Proposed reaction pathway for* N-'*amidination*' *of distamycin-NA*

While the N-terminus of the acetylated oligomers remained to a large degree intact under conditions of melting-curve analysis (see *Sect. 3*), the oligomers containing N-terminal amidine functions were transformed by hydrolysis to the corresponding formamide derivatives. The transformations of the polyamides **7c** and **8c** into their formamide derivatives **7d** and **8d** were documented by reversed-phase HPLC and MALDI-TOF-MS (*Figs. 8* and *9*). The mass analysis of the newly formed products showed a mass difference of 28.6 ± 0.8 dalton between the *N*-unmodified and the newly formed polyamide, which is in accord with the *N,N*-dimethylformamidine function being hydrolyzed to a formamide function. This transformation is accompanied with a loss of polarity as it can been seen of the newly formed peak with higher retention time in the HPLCs. This kind of hydrolysis is well-known to occur in acidic to alkaline environments for aromatic amidines in general [12] and for the netropsin in particular [13].

2.4. *Attempts to Improve Solid-Phase Synthesis.* In a further attempt, the synthesis was repeated with two main alterations. The amino-acid building block **2** was produced without AcOH by changing the workup procedure, and for the activation of **2**, no DCC excess was used anymore. Furthermore, the symmetric anhydride was isolated as a white powder and used as such in subsequent coupling reactions. With this procedure, the synthesis of a decamer distamycin-NA was attempted. The observed coupling efficiencies determined by UV spectroscopy as described above indicated a rapid breakdown of the chain-extension reaction. After ten coupling steps, the polyamides were detached from the solid support and analyzed, as described before. The HPLC and MALDI-TOF-MS analysis of the crude material revealed that the chain elongation was inhibited again by N-terminal 'amidination' of intermediates. The HPLC chromatogram in *Fig. 10* shows four distinct peaks attributable to **5c**–**8c** indicating chain-growth abortion after the tetrameric level.

3. UV-Melting-Curve Analysis. – The isolated trimer and tetramer polyamides **7b**, **7c**, and **8c** were subjected to standard UV-melting-curve analysis (H_2O, 100 mM NaCl,

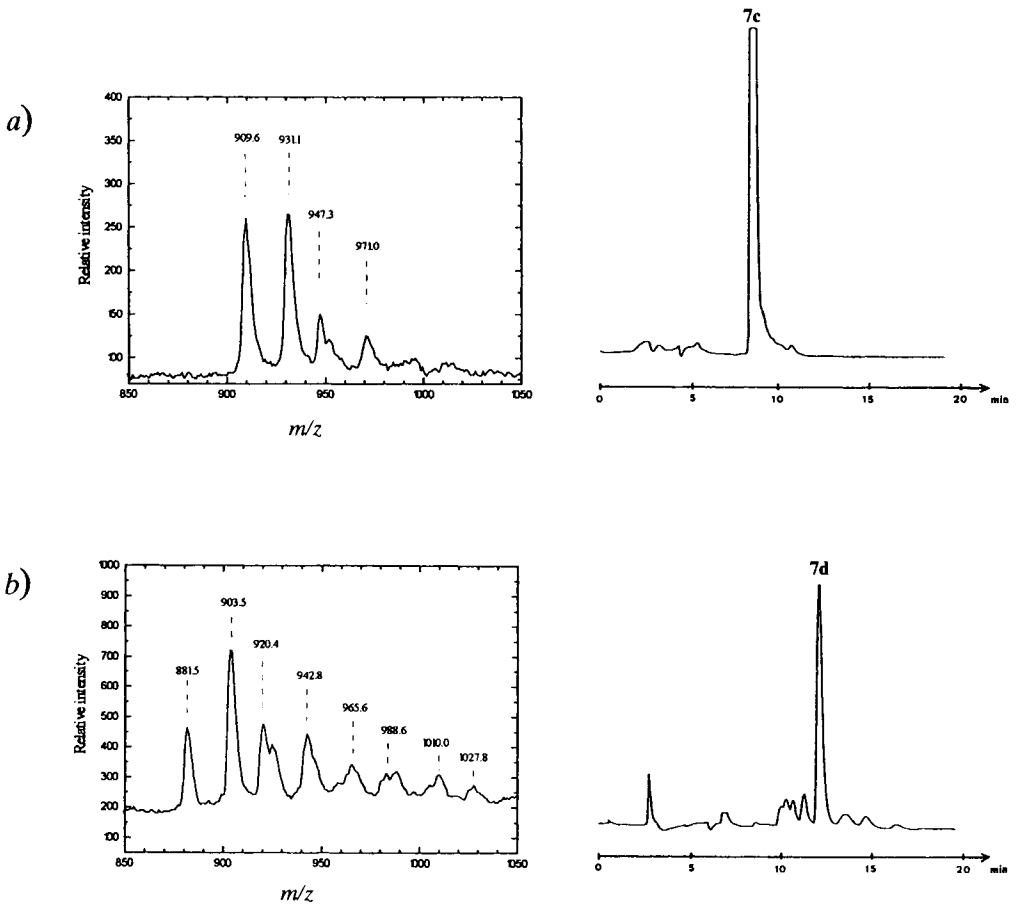


Fig. 8. a) *MALDI-TOF Mass spectrum of the corresponding HPLC fraction* **7c** *and* b) *HPLC of* **7c** *after aqueous hydrolysis and MALDI-TOF mass spectrum of the isolated fraction* **7d**. HPLC Conditions: *Nucleosil-CC-250/4-5-C18* column (*Macherey & Nagel*); eluent *A*: 0.1 % CF_3COOH in H_2O; eluent *B*: MeCN/H_2O 9:1; *A*/*B* 85:15 (*v*/*v*); flow rate 1 ml/min; UV detection at 260 nm. Hydrolysis conditions: H_2O, 100 mM NaCl, 10 mM Na_2HPO_4, pH 7.0, 0–100°.

10 mM Na_2HPO_4, pH 7.0, 0–100°) [14]. As oligonucleotide single strands, we chose $d(A_{10})$ and poly(rA) in a stoichiometric 1:1 base/base ratio. No cooperative melting behavior of these mixtures, reminiscent of a denaturing process, was observed in both cases. This indicates that the chain length of the distamycin-Nas may still be too short to effect efficient pairing to complementary DNA or RNA in the micromolar range [15].

4. Conclusion. – The protected amino-acid building block **2** was used in the Fmoc-peptide-synthesis protocol on a lysine-derivatized *Wang* resin. During oligomerization to a hexameric distamycin-NA, unwelcome side reactions occurred and produced a mixture of N-terminal-modified distamycin-NA derivatives with various chain lengths. With the aid of combined HPLC and MALDI-TOF-MS analysis, a series of oligoamides carrying

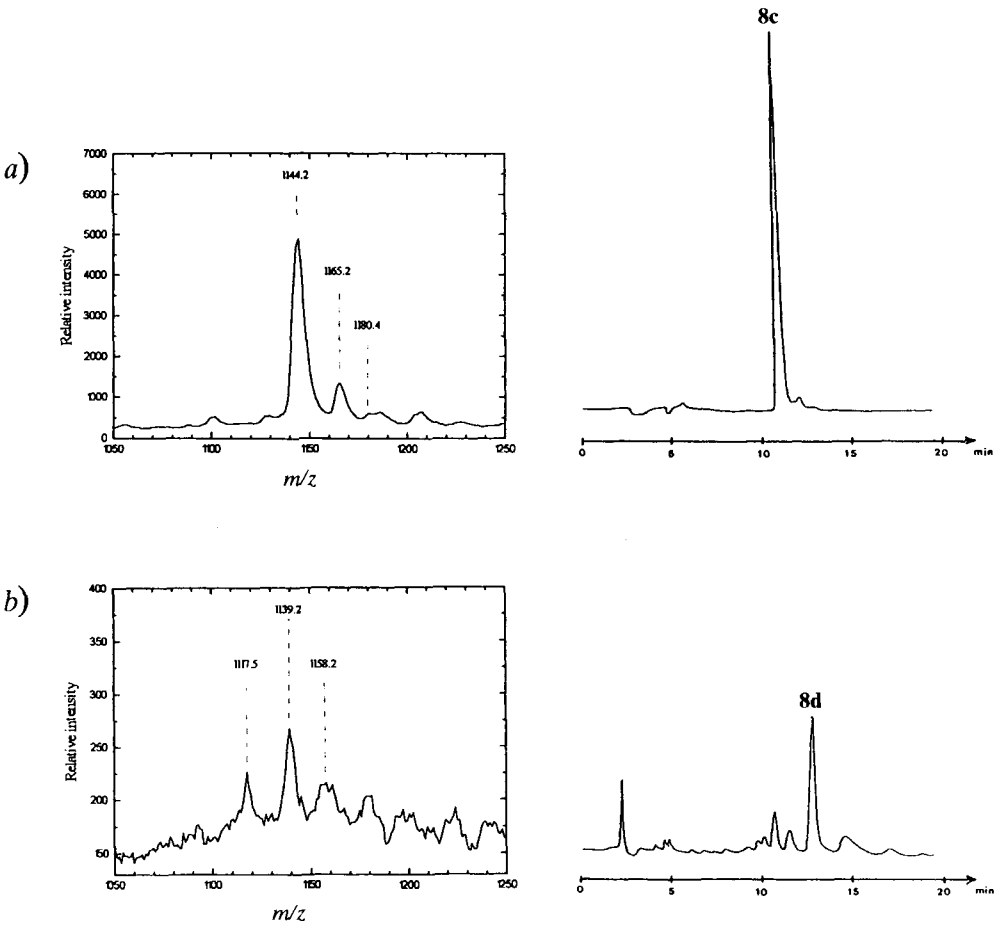


Fig. 9. a) *MALDI-TOF Mass spectrum of the corresponding HPLC fraction* **8c** *and* b) *HPLC of* **8c** *after aqueous hydrolysis, and MALDI-TOF mass spectrum of the isolated fraction* **8d**. HPLC and hydrolysis conditions, see *Fig. 8*.

acetamide- and *N*,*N*-dimethylformamidine-functionalized N-termini were isolated in pure form and characterized. Acetylation occurred as a result of AcOH being associated with preparation of **2** in small quantity (3–7%, ^{1}H-NMR), which showed competitive advantage in the coupling reaction, whereas the formamide function was attributed to arise from activated solvent DMF. Both reactions thus prevented the synthesis of longer sequences. In an attempt to optimize oligomerization, the acetylation was prevented by exclusion of AcOH in the preparation of the building block **2**, and excess of the activating agent DCC for *in situ* preparation of the anhydride was avoided. Nevertheless, 'amidination' could not be suppressed and again aborted the chain growth. This side reaction can, in principle, be ruled out by replacing the solvent DMF in the coupling reaction. This, however, needs careful screening of alternative solvents since the solubility of the anhydride of **2** is poor in most organic solvents. Taken these measures, it should be possible

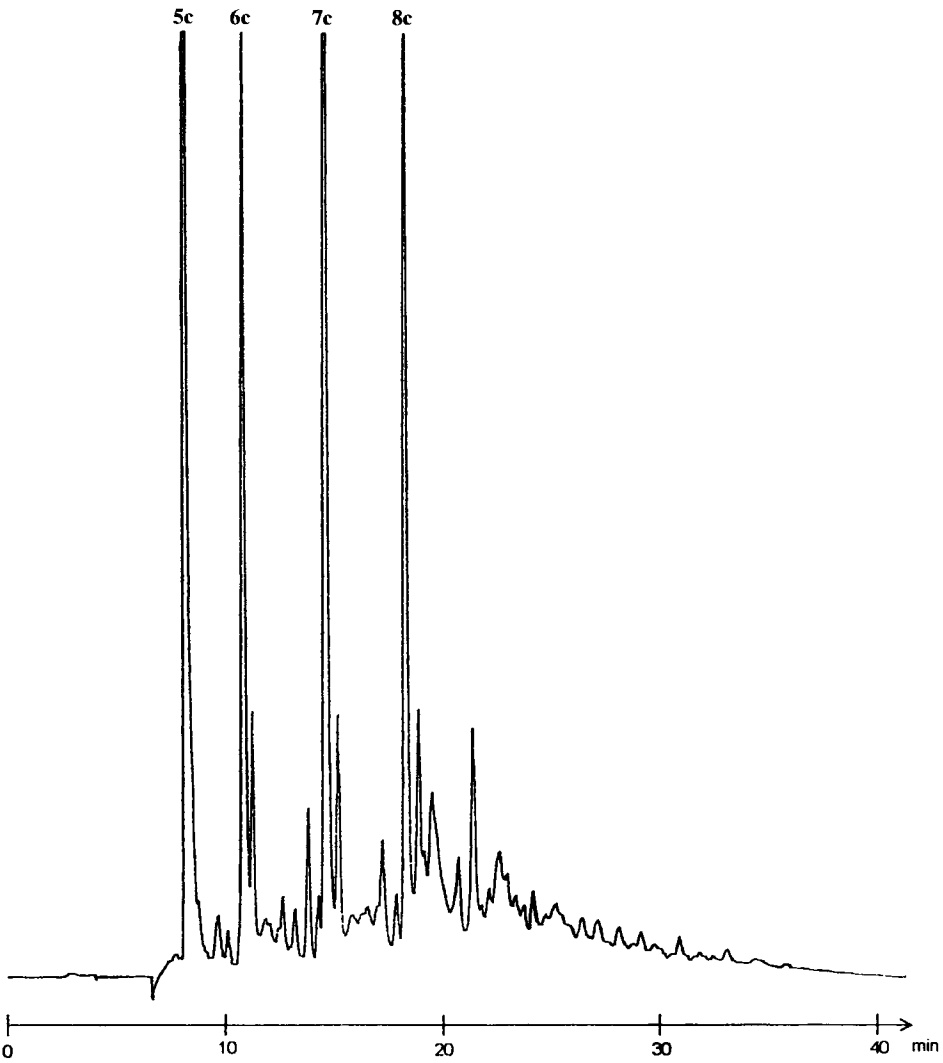


Fig. 10. *HPLC Analysis of the crude mixture from modified solid-phase synthesis.* HPLC Conditions as described in *Fig. 4*, except for linear gradient: 10–30% *B* in 35 min; assignments by comparison with the known t_R values and by MALDI-TOF-MS analyses of the crude mixture.

to produce longer distamycin-NAs in the future. This is necessary in order to be able to definitely answer the question whether distamycin-NA is a true DNA analog or not.

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Experimental Part

General. For quality of solvents and reagents used, as well as for spectroscopic characterization of products, see [2]. Labeling of oligonucleotides by numbering the residues from the N- to the C-terminus; see also *Formula* **2**. Reagents and solvents for solid-phase synthesis: Fmoc-Lys(ε-Boc)-*Wang* resin, *Bachem*. DCC, DMF, piperidine, and CF_3COOH, *Fluka*, highest quality available. HPLC: *Pharmacia-LKB-2249* gradient system attached to an *ABI-Kratos-Spectroflow-757* UV/VIS detector and a *Tarkan W + W* recorder *1100*. MALDI-TOF MS: spectrometer home-built by *Schlunegger* and coworkers [16], cylindrical probe tip made of gold; ion acceleration to 28 keV in two stages during the laser irradiation, detector voltage usually between −4.0 kV and −4.5 kV; ion recording in the positive detection mode, average of signals from the individual laser shots (20–100 shots); if not otherwise stated, matrix 1% DHB (2,5-dihydroxybenzoic acid) in H_2O/MeCN 1:3; peaks in *m/z*.

4-{[(9H-Fluoren-9-ylmethoxy)carbonyl]amino}-3-(1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)thiophene-2-carboxylic Acid (**2**). To a vigorously stirred soln. of methyl 4-amino-3-(1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)thiophene-2-carboxylate (**1**; 0.6 g, 2.25 mmol) in dioxane/H_2O 4:1 (60 ml), 2M KOH (30 ml) was added and stirred for 10 min. By adding a soln. of sat. $NaHCO_3$ soln. (148 ml) and dioxane (111 ml), the pH was adjusted to pH 9–10, and a soln. of Fmoc-Cl (3.0 g, 11.6 mmol) in dioxane (30 ml) was added in four portions within 1.3 h.

Workup with AcOH: The resulting white suspension was poured to 10% AcOH (800 ml), the aq. soln. extracted with AcOEt (3 × 600 ml), and the combined org. phase concentrated to 1/5 of the volume. EtOH (50 ml) and toluene (100 ml) were added and coevaporated. The resulting yellow oil (5.2 g) was dissolved in MeOH (250 ml) and adsorbed on silica gel (52 g). Purification by FC ($CHCl_3$/MeOH/AcOH 9.5:0.25:0.25, then 8.5:1.0:0.5) furnished **2** as a white powder containing 15% AcOH (^{1}H-NMR). Additional coevaporation with toluene (2 ×) reduced the content of AcOH to 3–7% in **2** (0.995 g, 93%; yield not corrected).

Workup without Chromatography: The suspension was poured to 1N HCl (400 ml), the aq. soln. extracted with $CHCl_3$ (3 × 250 ml), and the combined org. layer washed with 1N HCl (200 ml) and evaporated. The oily residue was taken up in dioxane (5 ml), and Et_2O (400 ml) was added. After standing at 4° overnight, **2** (0.995 g, 93%) precipitated as anal. pure white powder. TLC ($CHCl_3$/MeOH/AcOH 8.5:1:0.5): R_f 0.55. UV/VIS (MeCN): 204 (83900), 259 (33800, br.), 288 (11500) 298 (11700). IR (KBr): 3434*m*, 3252*m*, 3065*m*, 1695*s*, 1558*m*, 1450*m*, 1409*m*, 1319*m*, 1286*m*, 1226*s*, 1105*w*, 1079*m*, 982*w*, 876*w*, 811*w*, 760*m*, 741*m*, 621*w*, 590*w*. ^{1}H-NMR (see Formula **2**): 4.30 (br. *t*, 1 H); 4.37–4.55 (*m*, 2 H); 5.63 (*dd*, *J* = 7.9, 1.7, H–C(5)); 7.28–7.47 (*m*, 4 arom. H, H–C(6)); 7.66–7.73 (*m*, 2 arom. H); 7.75–7.92 (*m*, 2 arom. H, H–C(5′)); 10.03 (*s*, HN–C(4′)); 11.34 (*d*, *J* = 1.5, H–N(3)). ^{13}C-NMR: 46.74 (*d*); 66.47 (*t*); 101.93 (*d*); 114.56 (*d*); 120.41 (*d*); 125.38 (*d*); 127.41 (*d*); 127.96 (*d*); 128.64 (*s*); 131.52 (*s*); 134.56 (*s*); 140.97 (*s*); 143.82 (*s*); 145.85 (*d*); 150.55 (*s*); 154.15 (*s*); 161.42 (*s*); 164.64 (*s*). MALDI-TOF-MS (Calc. for M^+: 475.48): 476.5 ($[M + H]^+$), 498.7 ($[M + Na]^+$). Anal. calc. for $C_{24}H_{17}N_3O_6S \cdot 0.5\,H_2O$ (484.85): C 59.45, H 3.74, N 8.67, O 21.44; found: C 59.81, H 4.04, N 8.29, O 21.61.

NH_2-(ThU)-Lys(ε-Boc)-OMe (**3**). To a soln. of **2** (42 mg, 88 μmol) and BtOH (14 mg, 88 μmol) in DMF (0.4 ml), DCC (44 mg, 213 μmol) was added. To the newly formed suspension, H-Lys(ε-Boc)-OMe · HCl (26 mg, 89 μmol) in DMF (0.2 ml) with Et_3N (13 μl, 89 μmol) was added. After stirring for 1.5 h under Ar, the suspension was filtered over glass wool which was washed with dioxane/H_2O 4:1 (4 ml). The filtrate was evaporated and the crude material dissolved in dioxane (1 ml) and piperidine (0.5 ml) and stirred for 20 min. After evaporation of the volatile components, the residue was purified by flash chromatography (FC) (EtOH/CH_2Cl_2/25% NH_3 soln. 0.5:10:0.05): **3** (29 mg, 67%). White powder. TLC (MeOH/CH_2Cl_2/25% NH_3 soln. 1:9:0.05): R_f 0.18. UV/VIS (H_2O): 200 (21200), 257 (13200), 308 (sh, 4500). IR (KBr): 3352*m*, 2950*m*, 2931*m*, 1694*s*, 1520*s*, 1480*m*, 1441*m*, 1366*m*, 1281*s*, 1171*s*, 1025*m*, 868*w*, 810*m*, 750*m*, 668*m*, 624*w*, 549*w*. ^{1}H-NMR: 1.20–1.38 (*m*, 4 H); 1.35 (*s*, 9 H), 1.60–1.75 (*m*, 2 H); 2.80–2.90 (*m*, 2 H); 3.60 (*s*, 3 H); 4.15–4.30 (*m*, 1 H); 5.00 (*s*, NH_2–C(4′.1)); 5.58, 5.59 (*d*, *J* = 8.1, *J* = 8.1, H–C(5.1)); 6.34, 6.35 (*s*, H–C(5′.1)); 6.76 (br. *t*, HN–C(6.2)); 7.28, 7.29 (*d*, *J* = 8.1, 8.1, H–C(6.1)); 8.17, 8.24 (*d*, *J* = 7.4, 7.7, HN–C(2.2)); 11.29 (*s*, H–N(3.1)). ^{13}C-NMR: 23.25 (*t*); 28.74 (*q*); 29.45 (*t*); 30.57 (*t*); 40.0 (*t*); 52.49, 52.84 (*d* and *q*); 78.19 (*s*); 100.11, 100.29 (2 *d*); 102.14, 102.34 (2 *d*); 128.57 (*s*); 129.95 (*s*); 144.32 (*s*); 146.30, 146.50 (2 *d*); 150.72 (*s*); 156.26 (*s*); 161.02 (*s*); 164.76 (*s*); 172.78 (*s*). MALDI-TOF-MS: 518.6 ($[M + Na]^+$, calc. 495.56).

Fmoc-NH(ThU)$_2$-COOMe (**4**). To a soln. of **2** (93 mg, 0.20 mmol) in DMF (0.3 ml), DCC (48 mg, 0.23 mmol) was added. After stirring for 0.5 h under Ar, the resulting suspension was filtered over glass wool and washed with DMF (2 × 0.1 ml). Solid **1** (27 mg, 0.10 mmol) was added to the filtrate. The mixture was stirred vigorously for 6 h and quenched with dioxane/H_2O 4:1 (4 ml). The solvents were evaporated. The resulting oil (185 mg) was dissolved in dioxane/H_2O/acetone 12:3:5 (20 ml) and the soln. adsorbed on silica gel (2.5 g). Purification by FC (EtOH/CH_2Cl_2 0.3–0.5:10) yielded **4** (40 mg, 55%). White powder. TLC (MeOH/CH_2Cl_2 17:9) R_f 0.58. ^{1}H-NMR: 3.75 (*s*, MeOOC–C(2′)); 4.30 (br. *t*, 1 H); 4.38–4.52 (*m*, 2 H); 5.57–5.68 (*m*, 2 H,

H–C(5)); 7.30–7.48 (*m*, 6 H, 4 arom. H, 2 H–C(6)); 7.65–7.80 (*m*, 3 H, 2 arom. H, H–C(5')); 7.86–7.93 (*m*, 2 arom. H); 8.15, 8.17 (2 *s*, 1 H, H–C(5')); 9.94 (*s*, NH); 10.47 (*s*, NH); 11.29 (*s*, NH); 11.39 (*s*, NH). MALDI-TOF MS (calc. for M^+: 724.73): 726.5 ($[M + H]^+$), 747.3 ($[M + Na]^+$), 763.5 ($[M + K]^+$).

Solid-Phase Synthesis. Activation of **2** *for Hexamer Synthesis.* To a soln. of **2** (containing 3–7% AcOH) in DMF (0.3 ml), DCC (1.2 equiv.) was added. After stirring for 0.5 h under Ar, the resulting suspension was filtered over glass wool to the swollen resin. The residue on the filter was washed with DMF (2 × 0.1 ml) resulting in 0.5 ml of activated **2**. *Soln. A*: 400 mM of **2**. *Soln. B*: 260 mM of **2**.

Activation of **2** *for Decamer Synthesis.* To a soln. of anal. pure **2** (0.93 g, 1.95 mmol) in dioxane (15 ml), DCC (0.48 equiv.) was added in dioxane (2 ml). After stirring for 3.5 h under Ar, the resulting urea was filtered off and the filtrate evaporated. The resulting white powder (1.0 g, essentially symm. anhydride of **2**) was stored in an exsiccator. *Soln. C*: 100 mg of the symm. anhydride of **2** was dissolved in DMF (0.5 ml) directly before use.

Manual Synthesis Protocol (see *Scheme 2*). FmocNH-Lys(ε-Boc)-*Wang* resin (50 mg, 0.033 mmol) was placed in a 9-ml glass reaction vessel of a bubbler system for manual synthesis as described in [10] and shaken in DMF for 30 min. After draining the reaction vessel, the resin was washed with DMF (5 × 1 min) and the Fmoc group removed with 20% piperidine in DMF (1 × 3 min, 1 × 7 min). The resin was washed with DMF (10 × 1 min), and an aliquot of the deprotection soln. was taken for determination of the UV absorbance at 267 nm. To the completely drained vessel, activated monomer was added (*Soln. A*, *B*, or *C*; for details, see *Table 2*). The reaction mixture was agitated by gently bubbling N_2 through the slurry. After this, the vessel was drained and the solid phase washed with DMF (5 × 1 min), and the coupling reaction was repeated by adding activated monomer again (*Table 2*). The vessel was drained completely and washed with DMF (10 × 1 min). The resin was ready for another deprotection/coupling cycle. After the last coupling step, followed by the final Fmoc removal and resin washing as described above, the vessel was drained completely. The polyamides were detached with CF_3COOH/H_2O 95:5 (1 × 2 h, 1 × 1 min, 1 × 1 min, 1 × 1 min). The solvents were evaporated completely, and the faint yellow solid was dissolved in H_2O for further HPLC and MALDI-TOF MS analysis (see *Sect. 2.3*).

Table 2. *Summary of the Coupling Data Used on Solid-Phase Synthesis of Hexamer and Decamer*

	Hexamer											Decamer	
Coupling step	1	2		3		4		5		6		1–10	
Soln.	*A*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*C*	*C*
Time of coupling	3 h	19 h	8 h	19 h	19 h	23 h	19 h	23 h	23 h	23 h	23 h	13 h	9 h

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