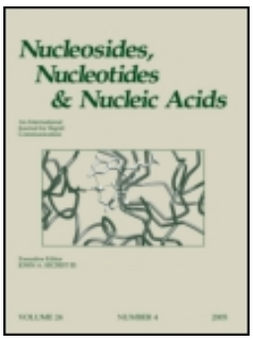

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THE MECHANISM OF CLEAVAGE UNDER BASIC CONDITIONS OF SUCCINYL-ANCHORED OLIGONUCLEOTIDES

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Abstract. Studies with a model compound provide direct evidence that cleavage of succinyl-anchored oligonucleotides takes place by intramolecular nucleophilic attack of the conjugate base of the succinamide at the ester carbonyl group. An N-substituted succinimide is exclusively formed with piperidine, DBU and TBAF, but when ammonia is used this general mechanism seems to coexist with ammonolysis of the succinate ester. Cleavage with TBAF is extremely fast.

As a part of our ongoing research directed towards the development of a method for solid-phase oligoribonucleotide synthesis using the base-labile Fmoc group for the protection of the 5'-OH function[1], we were interested in studying the reasons for the lability of the succinyl linker under basic conditions. In the same context, other authors have found that the succinyl linker is labile to bases such as DBU and piperidine[2-4]. To completely avoid the premature cleavage during chain elongation we have introduced an acid-labile linker[1] whereas sufficient resistance to DBU has been achieved using a succinyl-sarcosyl linker[4,5]. A linker with the succinyl group attached to a different secondary amine shows the same stability[6]. This finding provides indirect evidence of the cleavage mechanism of the succinyl linker, which is presumed to involve deprotonation of the succinamide nitrogen followed by intramolecular nucleophilic displacement at the ester carbonyl group[4].

This is certainly the most plausible mechanism when a non-nucleophilic base like DBU is used, but there is some controversy as to whether it also applies when a nucleophilic base is employed. It should be borne in mind that the cleavage of the succinyl-sarcosyl linker, which cannot be deprotonated, is performed with concentrated aqueous ammonia in

less than one hour at room temperature, that is, under the same conditions as the succinyl linker using the standard method for solid-phase oligonucleotide synthesis[7].

To gain some insight into the mechanism of cleavage we have synthesized the model compound **1**[8] (Figure 1), and the progress of its reaction with different bases has been monitored by HPLC. The structure of **1** reproduces that of a nucleoside attached through a succinyl linker to the aminomethyl-polystyrene resin that we are currently using in our research[9]. If the cleavage of **1** under basic conditions takes place following the suggested mechanism it would produce 5'-O-DMT-thymidine **2** and N-benzylsuccinimide **3**. Nucleophilic attack at the ester carbonyl group by aqueous ammonia could produce N-benzylsuccinamide **4** and/or N-benzylsuccinamic acid **5**. Therefore **3**, **4** and **5** have been obtained by simple, well known chemical procedures[10] and used as standards in the HPLC study[11]. In addition to DBU, piperidine and aqueous ammonia, we have also studied the reaction of the model compound **1** with tetrabutylammonium fluoride (TBAF) since it is well known that fluoride anion is a strong base and, furthermore, promotes intramolecular cyclizations of carbamates[12].

The results of this study, in terms of disappearance of compound **1** at different reaction times, are shown in Table 1.

It should be stressed that the fastest reaction takes place with TBAF and that it is complete in less than 5 minutes, thus being an alternative reagent for the rapid cleavage of succinyl-anchored oligonucleotides. The cleavage rates with DBU and piperidine correlate well with their basicity (pK_a of BH^+ are 12.5 and 11.1 respectively). However, the data in Table 1 correspond to the cleavage of a small model compound in solution, and the solid-phase reaction rates must be lower, particularly if an oligonucleotide, instead of a nucleoside, is anchored to the solid support. For instance, our experience indicates that it is hard to quantitatively recover an oligonucleotide with DBU treatment: 30% cleavage of an heptanucleotide was observed after a 5 day treatment with 0.5M DBU solution in pyridine[13]. Likewise, complete cleavage with TBAF of a hexanucleotide attached to CPG was only achieved in 30 minutes[14].

From the mechanistic point of view, the nucleoside **2** and the succinimide **3** are the products formed in DBU, piperidine and TBAF reactions, which proves that when the base is non-nucleophilic or a poor nucleophile, the succinyl linker is cleaved by intramolecular attack of the conjugate base of the succinamide function to the ester carbonyl group (Figure 2).

The formation of **2**, **4** and **5**, but not **3**, with conc. ammonia seems to indicate that the cleavage proceeds by direct nucleophilic attack of the reagent at the carbonyl of the ester. However, if the succinimide **3** were formed at the first stages of the reaction, it could also react with ammonia to give **4** and **5**. Therefore, another set of experiments was designed to

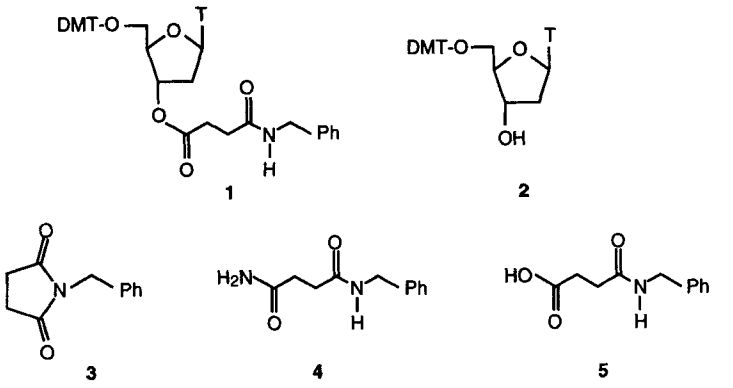

FIGURE 1. Structures of the model compounds used in this study.

TABLE 1. Cleavage of compound **1** with different bases.

Time	10% piperidine/DMF	0.1M DBU/THF	0.01M TBAF/THF	conc.aq.NH_3
5 min	-	-	100%	97%
1 h	15%	19%	-	100%
8 h	58%	82%	-	-

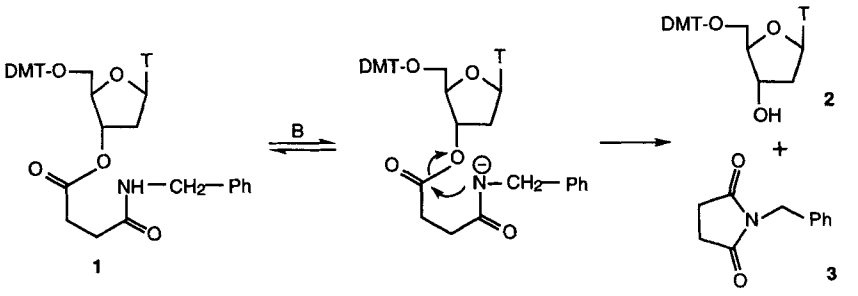

FIGURE 2. Mechanism of the base-promoted cleavage of succinyl-linked nucleoside **1**.

TABLE 2. Ratio (%) of products when **1**, **3** and **4** are treated with conc. aq. NH_3

		Reaction products		
Reaction	Time	**3**	**4**	**5**
1 + NH_3*	2 min	15	79	6
	5 min	0	91	9
	1 h	0	86	14
3 + NH_3	2 min	30	58	12
	5 min	0	82	18
	1 h	0	75	25
4 + NH_3	1 h	-	96	4
	2 h	-	88	12
	4 h	-	76	24
	8 h	-	60	40
	24 h	-	15	85

*In this case, **2** is obviously detected (and **1** at 2 and 5 min), but the ratio is given so that **3** + **4** + **5** = 100%

evaluate the stability of **1**, **3**, **4** and **5** to ammonia treatment. The most significant results are shown in Table 2. Compound **5** was shown to be stable to the reaction conditions.

From the results shown in Table 2 it is clear that the succinimide **3** is formed in the reaction of **1** with ammonia but it decomposes very quickly ($t_{1/2}$=1min) to give the amide **4** and the acid **5**. After the first minute of reaction (data not shown in Table 2), almost 40% of **3** is present in the reaction mixture of **1** with NH_3, and only 54% of the starting material remains unaltered when **3** is treated with NH_3. The hydrolysis of the amide **4** to give the acid **5** takes place very slowly ($t_{1/2}$=10h).

However, the remaining question is if the amide **4** detected at the first stages of the reaction comes only from the decomposition of **3** or it is also formed by direct ammonolysis of the succinate ester of **1**. In our opinion, the analysis of the data in Table 2 indicates that both mechanisms coexist, since in 5 minutes more amide **4** is formed when **1** is the starting material (91%) than when **3** alone is treated with ammonia (82%). The same is also true for the 2 min treatment without considering the amount of **3** still present in the reaction mixture, since 93% and 83% of **4**/(**4**+**5**) are found in the reactions of **1** and **3**, respectively.

The question of whether some of the acid **5** is also formed by direct hydrolysis of **1** cannot be answered, but it is probable that in concentrated aqueous ammonia ammonolysis largely predominates over hydrolysis (see the results of **3** + NH_3).

In summary, we have demonstrated that, in most cases and despite the nucleophilic character of the base, the cleavage of the succinyl linker takes place by a nucleophilic attack of the conjugate base of the succinamide function at the ester carbonyl group to give an N-substituted succinimide derivative. We have found direct evidence of the formation of this succinimide, which is the only product formed when the base is not a good nucleophile. When ammonia is used as the cleavage reagent this general mechanism coexists to some extent with the ammonolysis of the succinate ester. Cleavage of the succinyl linker with TBAF is extremely fast and may provide an alternative for the detachment from the solid support of oligonucleotides bearing ammonia-sensitive base analogues.

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8. Preparation of **1**: 0.14g of *p*-nitrophenol and 0.52g of DCC was added to a solution 0.64g of 5'-O-DMT-thymidine-3'-O-succinate in 4mL of dioxane and 0.2mL of pyridine. After 3h the reaction mixture was filtered, washed with dioxane and 0.14mL benzylamine in 1mL TEA and 0.14mL DMF was added to the filtrate. 2h later the reaction mixture was filtered, and the filtrates were evaporated to dryness, dissolved in AcOEt and washed with 5% citric acid, sat. aq. $NaHCO_3$ and brine. The organic phase residue was purified by silica gel flash column chromatography eluting with DCM/MeOH 97:3 (+0.5% TEA). The desired fractions were collected and evaporated to dryness (41% yield). Rf=0.24 (DCM/MeOH 95:5). m.p. 88-90°C. MS (FAB+) m/z: 733.5 $[M+H]^+$, 756.4 $[M+Na]^+$.

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10. **3** was obtained according to a described procedure: A.Arcoria, J.Barassin, H.Lumbroso *Bull. Soc. Chem. Fr.* **1963**, 2509-2514. Rf=0.68 (AcOEt/hexanes 9:1). m.p. 99-100°C. MS (CI, CH_4) m/z: 190 $[M+H]^+$, 218 $[M+C_2H_5]^+$, 230 $[M+C_3H_5]^+$.

Preparation of **4**: 0.6g of *p*-nitrophenol and 2.22g DCC[15] was added to a solution of 0.5g of succinamic acid in 6mL of DMF, 0.7mL of pyridine and 10mL DCM. The reaction mixture was vigourously stirred for 18h. The precipitate was filtered and washed with 4mL DCM. 0.60mL Benzylamine and 4.3mL TEA was added to the solution and the mixture was stirred for 18h. The precipitate was filtered, washed with DCM and crystallized in MeOH (28% yield). Rf=0.14 (DCM/AcOH 9:1). m.p. 194-195°C . MS (CI, CH_4) m/z: 207 $[M+H]^+$, 235 $[M+ C_2H_5]^+$, 247 $[M+C_3H_5]^+$.

Preparation of **5**: 0.22mL of benzylamine and 0.34mL DIEA was added to a suspension of 0.22g succinic anhydride in 15mL of DCM. After 18h the solution was diluted with DCM and washed with 10% citric acid and brine. The organic phase was dried with Na_2SO_4, filtered and the solvent was removed. The residue was dissolved in the minimal amount of AcOEt and precipitated over hexanes (49% yield). Rf=0.29 (DCM/AcOH 9:1). m.p. 138-139°C . MS (EI) m/z: 207 ($M^{+\cdot}$).

11. Aliquots of the reaction mixture (1μmol/mL) were taken at different reaction times, neutralized (Dowex 50 WX4, H^+ form, for DBU; glacial AcOH for piperidine and NH_3; 0.01M triethylammonium acetate for TBAF) and analyzed by HPLC: Nucleosil C18 (10μm, 250x4.6mm), gradient elution from 20% ACN/H_2O to 100% ACN in 20min, 1mL/min, λ=260nm. Retention times: 15.9min (**1**), 14.0min (**2**), 9.4min (**3**), 5.3min (**4**), 4.4min (**5**).

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15. Abreviations used are as follows: DCC=N,N'-dicyclohexylcarbodiimide, DMT=4,4'-dimethoxytrityl, TEA=triethylamine, DMF=N,N-dimethylformamide, DBU=1,8-diazabicyclo[5.4.0]undec-7-ene, DCM=dichloromethane.