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USE OF LEVULINIC ACID IN THE PROTECTION OF OLIGONUCLEOTIDES *VIA* THE MODIFIED PHOSPHOTRIESTER METHOD: SYNTHESIS OF DECARIBONUCLEOTIDE U-A-U-A-U-A-U-A-U-A.

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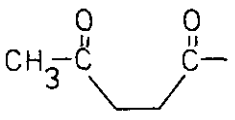
Several years ago Letsinger e.a.[1,2] introduced β-benzoylpropionyl as a protecting group in nucleic acid chemistry and showed that this group could be removed under neutral conditions; i.e. 0.5M hydrazine.hydrate in pyridine-acetic acid buffer (4:1 v/v) for 3 h. Unfortunately, the N-protecting benzoyl groups on cytidine and adenosine proved to be unstable under those conditions[3]. It is well known, however, that reactions initiated by nucleophilic attack, e.g. of hydrazine, on keto-functions, are much slower when the α-substituent is aryl instead of alkyl. The reason for this decreased reactivity has been attributed[4] to the loss of conjugation between the aryl and carbonyl functions in the transition state for hydrazine addition. We therefore selected levulinic acid (4-oxo-pentanoic acid) to replace β-benzoylpropionic acid as a possible protecting group.

For this reason we prepared model compound 1b (B=U)[5] by reaction of 1a (B=U)[6] (1 mmole) with levulinic acid (2 mmoles), DCCI (2 mmoles) and 4-dimethylaminopyridine[7] (10 mg) in dioxan (10 ml) solution (yield 96%). When 1b (0.1 mmole) was treated with 0.5M hydrazine.hydrate in pyridine-acetic acid (4:1 v/v, 1 ml), deblocking of the 5'-hydroxy function was complete within 2 min and 1a (B=U) could be recovered quantitatively. This experiment shows that levulinyl is a hundred times more labile than β-benzoylpropionyl as a hydrazine-labile protecting group.

Model compound 1b (B=U) was stable under standard reaction conditions in oligonucleotide synthesis *via* the modified triester method[8], i.e. mild reductive conditions (Zn-pyridine-acid) and during condensation reactions which involve arylsulphonyl-chlorides or -imidazolides. The half-times of hydrazinolysis, under the conditions as described above, of the N-protecting groups on cytidine and adenosine are 2½ h and 4 h respectively for N-benzoyl and 10 h and 16 h respectively for N-p-anisoyl. Furthermore, the phosphotriester moiety with 2-chlorophenyl as protecting group has a half-life of *ca.* 2 days. These findings indicate that the levulinyl group may be removed selectively in the presence of the other types of protecting groups.

These results were encouraging enough to make us try, whether the levulinyl group would also be useful in the synthesis of long oligonucleotides. To test this possibility, the synthesis of the decaribonucleotide U-A-U-A-U-A-U-A-U-A was undertaken.

Compound 2a (B=U)[5], prepared in 62% yield by the dropwise addition of a solution of DCCI (60 mmoles) in dioxan (50 ml) to a solution of 2'-O-(methoxytetrahydropyranyl)uridine[9] (20 mmoles), 1,2-dimethylimidazole (10 mmoles) and levulinic acid (60 mmoles) in 2,6-lutidine (7 ml) and dioxan (150 ml),was reacted together with 3[8], using 1-methylimidazole as a catalyst, to give 2b (B=U)[5] in 77% yield. Compound 2b ($B=A^{an}$)[5] was prepared analogously (38% yield over 2 steps). To remove the 5'-levulinyl protecting group a 1M solution of hydrazine.hydrate in pyridine-acetic acid (3:2 v/v, 30 ml) was added to a solution of 2b ($B=A^{an}$, 6 mmoles) in pyridine (30 ml). After



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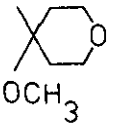

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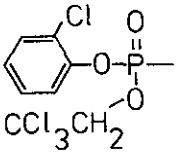

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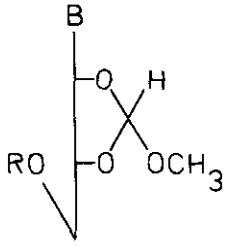
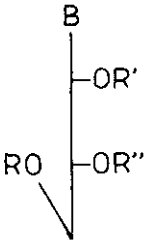
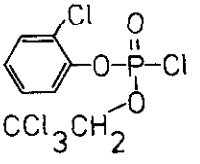


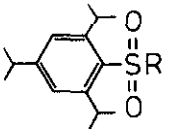
1 a: R = H.
b: R = I.
B = U, A^{an}.



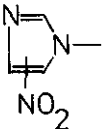
2 a: R = I, R' = II, R'' = H.
b: R = I, R' = II, R'' = III.
c: R = H, R' = II, R'' = III.
B = U, A^{an}.

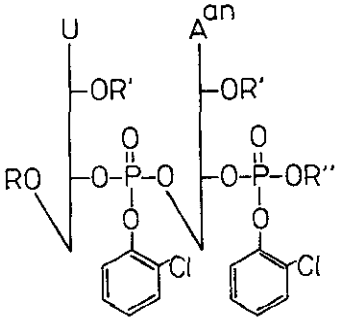


3

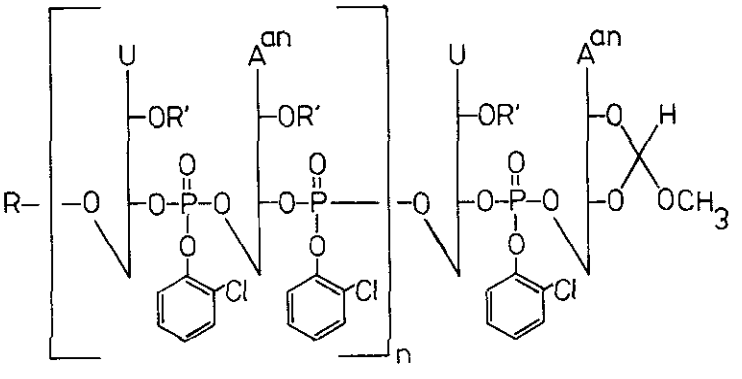


4 a: R = OH.
b: R =





5 a: R = I, R' = II, R'' = $-CH_2CCl_3$.
b: R = I, R' = II, R'' = H.



n = 0: 6.
n = 1: 7.
n = 2: 8.
n = 3: 9.
n = 4: 10.

a: R = I, R' = II.
b: R = H, R' = II.

U = Uracil-1-yl; A^{an} = 6-N-p-Anisoyladenin-9-yl.

2 min, the reaction was quenched by the addition, with cooling, of pentane-2,4-dione (6 ml). After work-up, 2c (B=A^{an})[5] was obtained as a homogeneous solid in 93% yield.

The crucial dinucleoside diphosphate 5a, which was used in each step of the subsequent block-condensations, was prepared from 2b (B=U) and 2c (B=A^{an}) by the modified triester method. Zinc (20 mmoles) was added to a solution of 2b (B=U, 2.3 mmoles) and TPSOH (4a, 0.6 mmole) in pyridine (20 ml). After 3 min, excess Zn was filtered off. The filtrate – after dilution with $CHCl_3$ (100 ml) – was washed with 1M triethylammonium bicarbonate (TEAB, 100 ml) and 0.01M TEAB (100 ml), and the organic layer was dried by repeated evaporation with anhydrous pyridine. To the resulting solution of the phosphodiester in pyridine (15 ml) was added 2c (B=A^{an}, 2 mmoles) and 2,4,6-triisopropylbenzenesulphonyl-4(5)nitro-imidazolide[5] (4b, TPSNI, 2.5 mmoles). Work-up of the reaction mixture after 16 h gave 5a in 87% yield. In the same way 6a was prepared in good yield by Zinc treatment of 2b (B=U) and subsequent coupling, using TPSNI (4b) as activating agent, with 1a (B=A^{an}). Removal of the levulinyl group from 6a with hydrazine afforded 6b as a homogeneous solid in 92% yield.

The usefulness of TPSNI, which is easily prepared starting from TPSCl[10] and 4(5)-nitroimidazole, should be pointed out in this study. In comparison with TPSCl it gives far less sulphonylation of the 5'-hydroxy function of the incoming nucleoside and, besides, a higher yield of the desired product is obtained. Furthermore, block-condensations between small and large oligonucleotides are much faster (two times) with TPSNI as condensing agent than with TPSCl. Recently, other reactive arylsulphonamides with lower[11,12] or similar[13] reactivity have been introduced and successfully applied.

Tetranucleotide 7a was prepared analogously as described above for the synthesis of the dinucleotide 5a. Thus, Zn-dust (10 mmoles) was added, with stirring, to a solution of 5a (0.72 mmole) and TPSOH (4a, 0.18 mmole) in pyridine (5 ml). After 3 min, excess Zn was filtered off and further processing afforded diester 5b. To the solution of 5b in pyridine (4 ml) was added 6b (0.6 mmole) and TPSNI (0.7 mmole). After 16 h, another portion of TPSNI (0.3 mmole) was added and after a total reaction time of 40 h, the mixture was worked up and chromatographed to give 7a as a homogeneous solid in 72% yield. The levulinyl group was removed by dissolving 7a (0.4 mmole) in pyridine (2 ml) and adding 1M hydrazine.hydrate in pyridine-acetic acid (3:2 v/v, 2 ml). After 4 min at 20°, the reaction was quenched by the addition, with cooling, of pentane-2,4-dione (4 mmoles). Work-up gave 7b in 93% yield. In the same way hexanucleotide 8a, octanucleotide 9a and decanucleotide 10a were prepared by Zinc treatment of 5a and subsequent condensation of resulting 5b with 7b, 8b and 9b respectively. Relevant data are given in Table 1.

Apart from the selective removal of the levulinyl group, we found three other properties which will make this group very attractive for further use: (i) despite its non-lipophilic character a reasonable drop in R_f value is observed upon its removal, which facilitates the monitoring of this deblocking step (see Table 1); (ii) the reaction time for deprotection is independent of the nature of the substrate[14]; this feature is a great improvement over the use of base labile protecting groups; in the latter case the reaction time depends on the sequence[15] as well as the lenght[16] of the oligonucleotides; (iii) the levulinyl group is stable under the conditions necessary for the removal of the phosphate protecting 2-chlorophenyl groups by fluoride ion; this property enables deblocking of the 2-chlorophenyl groups in triester intermediates bearing ester functions on the 5'-position.

TABLE 1

Synthesis of protected decanucleotides 10a and 10b.

3'-Phosphate component (mmoles)	5'-Hydroxy component (mmoles	Product (yield)[a]	R_f value[b]	Removal of the levulinyl group[c]			
				time	product	yield	R_f[b]
5b (0.72)	6b (0.60)	7a (72%)	0.46	4'	7b	93%	0.34
5b (0.45)	7b (0.325)	8a (69%)	0.45	4'	8b	80%	0.35
5b (0.25)	8b (0.125)	9a (74%)	0.40	3'	9b	81%	0.30
5b (0.15)	9b (0.05)	10a (61%)	0.32	4'	10b	76%	0.24

a) TPSNI (4b) was used in all condensation reactions; b) On S&S DC Fertigfolien F1500 LS254 in chloroform-methanol (92:8 v/v); c) Under the conditions as described in the text.

Thus, protected oligoribonucleotides 7a, 8a, 9a and 10a were completely deblocked by treatment with (a) 0.05M tetrabutylammonium fluoride in THF/pyridine/water (8:1:1 v/v/v), 3 eq. per phosphate moiety, for 16 h[16]; (b) 25% ammonia for 48 h; and, finally, 0.01N HCl (pH = 2) for 2 h, to give $(U\text{-}A)_2$, $(U\text{-}A)_3$, $(U\text{-}A)_4$ and $(U\text{-}A)_5$ respectively in good yields (80-95%, determined spectrophotometrically). The fidelity of the 3'-5' linkages was established by complete digestion of the products with Pancreatic RNAse, snake venom phosphodiesterase and spleen phosphodiesterase to the expected products in the correct ratios (deviations from the calculated ratios were less than 3%).

The levulinyl group has been used before as a protecting group, but the reported alkaline deprotection procedures, (a) sodium borohydride in dioxan-water for 20 min[17] and (b) hydrazine in boiling methanol for 3 h[18], are unsuitable for the use in oligonucleotide synthesis *via* phosphotriester intermediates.

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FOOTNOTES AND REFERENCES

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