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A NEW APPROACH TO THE SYNTHESIS OF OLIGONUCLEOTIDES AND THEIR PHOSPHOROTHIOATE ANALOGUES IN SOLUTION

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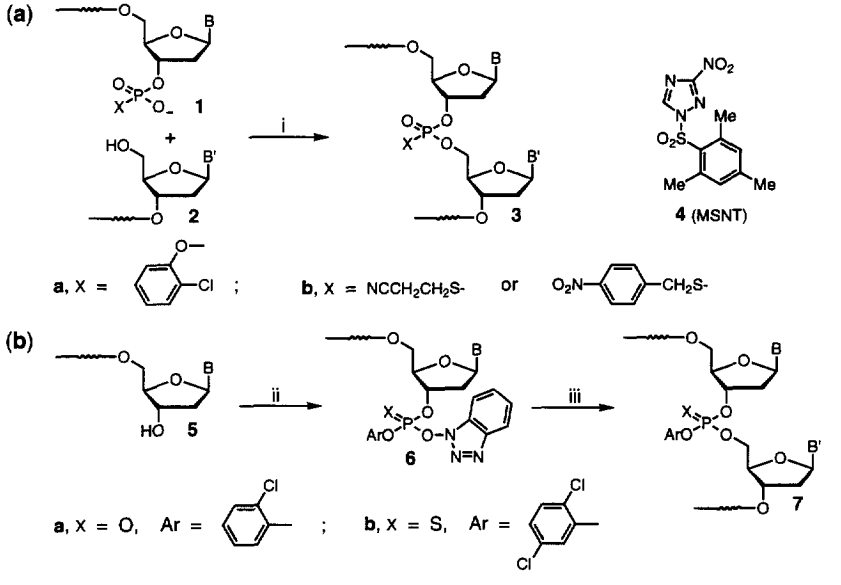
Abstract: A new approach to the synthesis of oligonucleotides and oligonucleotide phosphorothioates in solution is described; it is based on *H*-phosphonate coupling at - 40°C, followed by *in situ* sulfur-transfer with either *N*-[(4-chlorophenyl)sulfanyl]phthalimide **19** or 4-[(2-cyanoethyl)sulfanyl]morpholine-3,5-dione **21**.

Three main methods, namely the phosphotriester[1], phosphoramidite[2] and *H*-phosphonate[3] approaches have proved to be effective for the chemical synthesis of oligonucleotides. While the phosphotriester approach has been used most widely for synthesis in solution, the phosphoramidite and *H*-phosphonate approaches have been used almost exclusively in solid phase synthesis. The possible use of oligonucleotides and their phosphorothioate analogues in chemotherapy[4] has recently made their large scale synthesis a matter of considerable importance. Although the demand for relatively large quantities of material has so far been met mainly by the scaling-up of solid phase synthesis, we believe that, if a specific oligonucleotide sequence becomes licensed as a drug and multikilogram quantities of it are required, solution phase synthesis is likely to become the method of choice.



Scheme 1 *Reagents and conditions*: i, MSNT **4**, C_5H_5N, room temp., 30 min; ii, reagent prepared from 2-chlorophenyl phosphorodichloridate or 2,5-dichlorophenyl phosphorodichloridothioate, 1-hydroxybenzotriazole, base (C_5H_5N or Et_3N) and solvent (dioxane or THF), room temp.; iii, **2**, 1-methylimidazole, C_5H_5N.

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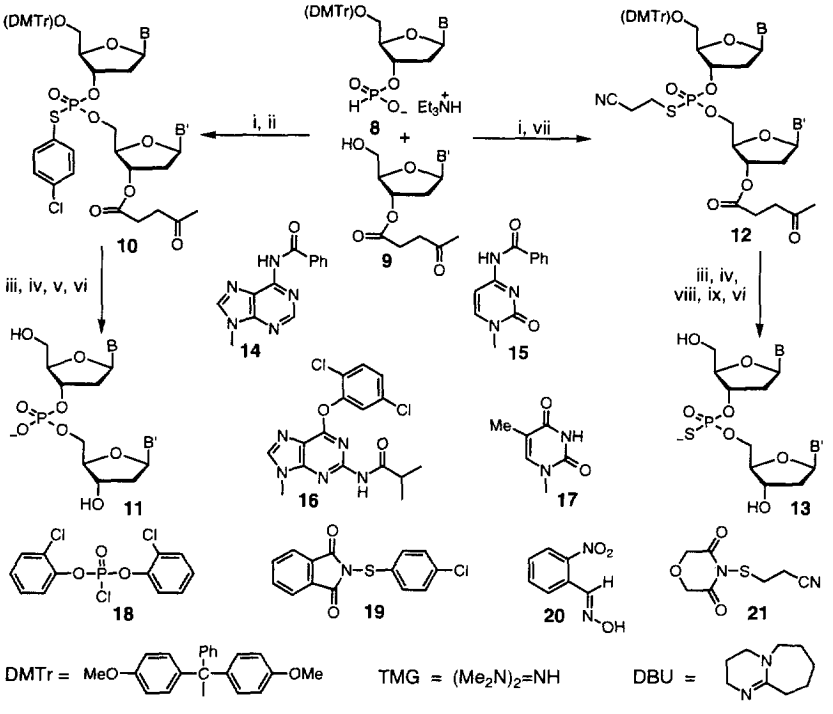
Two distinct synthetic strategies, which are indicated in outline in Scheme 1, have been applied successfully to the phosphotriester approach in solution. Perhaps the most widely used strategy for the synthesis of oligodeoxyribonucleotides in solution involves a coupling reaction between a protected nucleoside or oligonucleotide 3'-(2-chlorophenyl) phosphate[5] **1a** and a protected nucleoside or oligonucleotide **2** with a free 5'-hydroxy function to give a phosphotriester **3a**. A coupling agent such as 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-1*H*-triazole (MSNT)[6] **4** is required. This strategy has also been used in the synthesis of phosphorothioate analogues. Coupling is then effected in the same way between a protected nucleoside or oligonucleotide 3'-*S*-(2-cyanoethyl or, for example, 4-nitrobenzyl) phosphorothioate[7,8] **1b** and component **2**. The main disadvantages of this 'classical' phosphotriester approach are that some concomitant 5'-sulfonation (usually < 5%) of the second component **2** occurs[9], and that coupling reactions generally proceed relatively slowly. The sulfonation side-reaction both leads to lower yields and impedes the purification of the desired products. The second strategy for the synthesis of oligodeoxyribonucleotides in solution (Scheme 1b) involves the use of a bifunctional reagent derived from an aryl (usually 2-chlorophenyl) phosphorodichloridate and two molecular equivalents of 1-hydroxybenzotriazole[10], and the coupling reaction is assumed to proceed *via* intermediates of general structure **6a**. A related bifunctional reagent, derived from 2,5-dichlorophenyl phosphorodichloridothioate (Scheme 1b), has similarly been used[11] in the preparation of oligonucleotide phosphorothioates. The main disadvantages of the second strategy result directly from the involve-



Scheme 2 *Reagents and conditions*: i, **18**, C_5H_5N, CH_2Cl_2, -40°C, 5-10 min; ii, a, **19**, C_5H_5N, CH_2Cl_2, -40°C, 15 min, b, C_5H_5N - H_2O (1:1 v/v), -40°C to room temp.; iii, 4 M HCl / dioxane, CH_2Cl_2, -50°C, 5 min; iv, Ac_2O, C_5H_5N, room temp., 15 h; v, **20**, TMG, MeCN, room temp., 12 h; vi, a, conc. aq. NH_3 (d 0.88), 50°C,15 h, b, Amberlite IR-120 (plus), Na^+ form, H_2O; vii, a, **21**, C_5H_5N, CH_2Cl_2, -40°C, 15 min, b, C_5H_5N - H_2O (1:1 v/v), -40°C to room temp.; viii, DBU, Me_3SiCl, CH_2Cl_2, room temp., 30 min; ix, **20**, DBU, MeCN, room temp., 12 h.

ment of a bifunctional reagent. Thus the possibility exists of symmetrical coupling products being formed, and the presence of small quantities of moisture can lead to a significant diminution in coupling yields. We therefore set out to develop a new coupling procedure for the synthesis of oligonucleotides in solution that (a) is extremely efficient and does not lead to side-reactions, (b) proceeds relatively rapidly, and (c) is equally suitable for the preparation of oligonucleotides, their phosphorothioate analogues and chimeric oligonucleotides containing both phosphodiester and phosphorothioate diester internucleotide linkages.

The monomeric building blocks required in our new coupling procedure (Scheme 2) are triethylammonium 5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyribonucleoside *H*-phosphonates **8** (B = **14** - **17**) which can readily be prepared in almost quantitative yields from the corresponding protected nucleoside derivatives by our recently reported procedure[12]. When triethylammonium 6-*N*-benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyadenosine 3'-*H*-phosphonate[12] (DMTr-*A*p(H))[13] **8** (B = **14**), 4-*N*-benzoyl-3'-*O*-levulinyl-2'-deoxycytidine (HO-*C*-Lev)[13] **9** (B = **15**) and di-(2-chlorophenyl) phosphorochloridate[14] **18** were allowed to react together in pyridine - dichloromethane solution at - 40°C, the corresponding fully-protected dinucleoside *H*-phosphonate (DMTr-*A*p(H)*C*-Lev) was obtained apparently in quantitative yield within 5-10 min[17]. It is particularly noteworthy that a very high coupling efficiency was achieved with only *ca.* 20% excess of *H*-phosphonate monomer. No attempt was made to isolate the intermediate dinucleoside *H*-phosphonate (DMTr-*A*p(H)*C*-Lev). *N*-[(4-Chlorophenyl)sulfanyl]phthalimide[18] **19** was added to the reaction mixture[19] and the corresponding *S*-(4-chlorophenyl) dinucleoside phosphorothioate DMTr-*Ap(s')C*-Lev[13] **10** (B = **14**, B' = **15**) was obtained in *ca.* 99% isolated yield. Thus both the coupling and the sulfur-transfer[20] steps proceeded relatively quickly and virtually quantitatively at - 40°C.

In addition to the fact that it is carried out in homogeneous solution, the present coupling procedure differs from that followed in the *H*-phosphonate approach to solid phase synthesis[3] in at least two other important respects. First, it is carried out at a very low temperature. Side reactions which can accompany *H*-phosphonate coupling[22] are thereby avoided despite the fact that di-(2-chlorophenyl) phosphorodichloridate **18** rather than pivaloyl chloride[23] is used as the coupling reagent. Secondly, sulfur transfer is carried out after each coupling step rather than just once following the assembly of the whole oligomer sequence.

Hata and his coworkers had previously reported[24,25] a completely different approach to oligonucleotide synthesis by the phosphotriester approach in solution, based on *S*-phenyl phosphorothioate intermediates; van Boom and his coworkers also reported[26] an approach to oligonucleotide synthesis, based on *S*-(4-methylphenyl) phosphorothioate intermediates. Both research groups demonstrated[24-26] that unblocking with oximate ions[6,27] led to natural phosphodiester internucleotide linkages. We have confirmed that the unblocking of *S*-(4-chlorophenyl)-protected phosphorothioates with the conjugate base of *E*-2-nitrobenzaldoxime[27] **20** (oximate treatment) proceeds smoothly and with no detectable internucleotide cleavage. With regard to the overall unblocking strategy in oligodeoxyribonucleotide synthesis, another most important consideration is that the removal of the 5'-terminal DMTr protecting group ('detritylation') should proceed without concomitant depurination, especially of the 6-*N*-acyl-2'-deoxyadenosine residues. We have found that such depurination, which perhaps is difficult to avoid in solid phase synthesis, can be completely suppressed by effecting 'detritylation' with *ca.* 0.45 M hydrogen chloride in dioxane - dichloromethane (1 : 8 v/v) solution at -50°C[28]. Thus the four step procedure (Scheme 2, steps iii - vi) for the unblocking of DMTr-*Ap(s')C*-Lev **10** (B = **14**, B' = **15**) involves 'detritylation', acetylation of the 5'-terminal hydroxy function, oximate treatment, and finally treatment with concentrated aqueous ammonia to remove acyl protecting groups from the base residues and from the 3'- and 5'-terminal hydroxy functions. In this way, extremely pure d[ApC] **11** (B = adenin-9-yl, B' = cytosine-1-yl) was obtained *without further purification* and isolated as its sodium salt [Fig. 1a-c]. The monomeric building blocks **8** (B = **17**) and **9** (B' = **16**) were coupled together in the same way and on the same scale. After sulfur transfer with *N*-[(4-chlorophenyl)sulfanyl]phthalimide **19**, the fully-

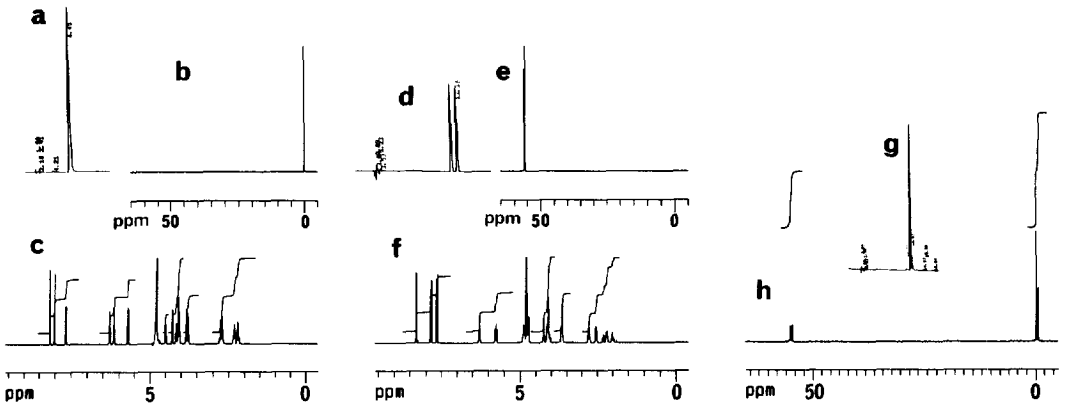
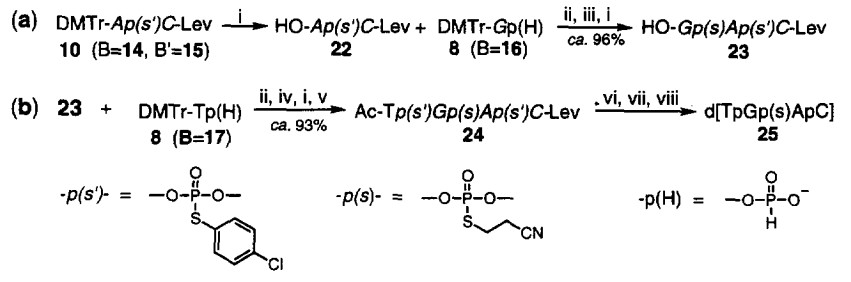


FIGURE 1. (a) reverse phase HPLC profile Na^+ d[ApC], (b) ^{31}P NMR spectrum (D_2O) of Na^+ d[ApC], (c) ^1H NMR spectrum (D_2O) of Na^+ d[ApC], (d) reverse phase HPLC profile of Na^+ d[Gp(s)A], (e) ^{31}P NMR spectrum (D_2O) of Na^+ d[Gp(s)A], (f) ^1H NMR spectrum (D_2O) of Na^+ d[Gp(s)A], (g) reverse phase HPLC profile of Na^+ d[TpGp(s)ApC], (h) ^{31}P NMR spectrum (D_2O) of Na^+ d[TpGp(s)ApC].

protected dinucleoside phosphorothioate DMTr-T*p(s')G*-Lev **10** (B = **17**, B' = **16**) was isolated in *ca.* 98% yield. Again, very pure d[TpG] **11** (B = thymin-1-yl, B' = guanin-9-yl) was obtained when this material was unblocked by the above procedure (Scheme 2, steps iii - vi).

The protocol for the preparation of fully-protected oligonucleotide phosphorothioates differs from that used for oligonucleotide synthesis[17,19] only in that sulfur-transfer is effected with 4-[(2-cyanoethyl)sulfanyl]morpholine-3,5-dione[29] **21** rather than with *N*-[(4-chlorophenyl)sulfanyl]phthalimide **19**. Triethylammonium 6-*O*-(2,5-dichlorophenyl)-5'-*O*-(4,4'-dimethoxytrityl)-2-*N*-isobutyryl-2'-deoxyguanosine 3'-*H*-phosphonate (DMTr-*G*p(H)) **8** (B = **16**) [*ca.* 4.8 mmol], 6-*N*-benzoyl-3'-*O*-levulinyl-2'-deoxyadenosine (HO-*A*-Lev) **9** (B' = **14**) [4.0 mmol] and di-(2-chlorophenyl) phosphorochloridate **18** [6.0 mmol] were allowed to react together in pyridine - dichloromethane solution at - 40°C for 5 - 10 min. 4-[(2-Cyanoethyl)sulfanyl]morpholine-3,5-dione **21** [8.0 mmol] (Scheme 2, step vii) was then added while the reactants were maintained at - 40°C. After 15 min, the products were worked up and fractionated by chromatography on silica gel to give the fully-protected dinucleoside phosphorothioate (DMTr-*Gp(s)A*-Lev) **12** (B = **16**, B' = **14**) in 99% isolated yield. This material was unblocked by a five-step procedure (Scheme 2, steps iii, iv, viii, ix and vi). Following the 'detritylation' and acetylation steps, the product was treated with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)[8] under strictly anhydrous conditions to remove the *S*-(2-cyanoethyl) protecting group. The 6-*O*-(2,5-dichlorophenyl) protecting group was then removed from the guanine residue by oximate treatment[30], and finally all of the acyl protecting groups were removed by ammonolysis. Extremely pure d[Gp(s)A] **13** (B = guanin-9-yl, B' = adenin-9-yl) was obtained *without further purification*, and was isolated as its sodium salt [Fig. 1d-f].

This new approach to the synthesis of oligonucleotides in solution is suitable for the preparation of sequences with (a) solely phosphodiester, (b) solely phosphorothioate diester and (c) a combination of both phosphodiester and phosphorothioate diester internucleotide linkages. The stepwise synthesis of d[TpGp(s)ApC] **25** which has one phosphorothioate diester and two phosphodiester internucleotide linkages is illustrated in outline in Scheme 3. All of the reactions involved were used above either in the preparation of d[ApC] **11** (B = adenin-9-yl, B' = cytosin-1-yl or of d[Gp(s)A] **13** (B = guanin-9-yl, B' = adinin-9-yl) (Scheme 2). First, the fully-protected dinucleoside phosphoro-



Scheme 3 *Reagents and conditions*: i, 4 M HCl / dioxane, CH_2Cl_2, -50°C, 5 min; ii, **18**, C_5H_5N, CH_2Cl_2, -40°C, 5-10 min; iii, a, **21**, C_5H_5N, CH_2Cl_2, -40°C, 15 min, b, C_5H_5N - H_2O (1:1 v/v), -40°C to room temp.; iv, a, **19**, C_5H_5N, CH_2Cl_2, -40°C, 15 min, b, C_5H_5N - H_2O (1:1 v/v), -40°C to room temp.; v, Ac_2O, C_5H_5N, room temp., 15 h; vi, DBU, Me_3SiCl, CH_2Cl_2, room temp., 30 min; vii, **20**, DBU, MeCN, room temp., 12 h. viii, a, conc. aq. NH_3(*d* 0.88), 50°C,15 h, b, Amberlite IR-120 (plus), Na^+ form, H_2O.

thioate DMTr-*Ap(s')C*-Lev **10** (B = **14**, B' = **15**) [*ca.* 0.75 mmol] was converted in four steps and in *ca.* 96% overall isolated yield (Scheme 3a) into the partially-protected trimer **23**[31]. This material was then coupled with DMTr-Tp(H) **8** (B = **17**) and the product was converted in three steps and in *ca.* 93% overall yield (Scheme 3b) into the fully-protected tetramer **24**. The latter material was unblocked to give d[TpGp(s)ApC] **25** which was isolated *without further purification* as its relatively pure (*ca.* 96.5% by HPLC) sodium salt [Fig. 1g-h]. The tetranucleoside triphosphate d[TpGpApC] and the tetranucleoside triphosphorothioate d[Cp(s)Tp(s)Gp(s)A] were also prepared by stepwise synthesis in very much the same way. The protocols followed differed from that outlined in Scheme 3 only inasmuch as the sulfur-transfer agent **19** was used exclusively in the preparation of d[TpGpApC] and the sulfur-transfer agent **21** was used exclusively in the preparation of d[Cp(s)Tp(s)Gp(s)A].

All of the fully-protected oligonucleotides described above (**10**, **12** and **24**) terminate in a 3'-*O*-levulinyl group. The latter protecting group may be removed by treatment with hydrazine under very mild conditions[32]. The resulting partially-protected oligonucleotides with free 3'-hydroxy functions may then be converted into the corresponding *H*-phosphonates which are the intermediates required for the block synthesis of oligonucleotides and their phosphorothioate analogues. Studies on block synthesis involving the latter intermediates are now being undertaken in our laboratory. We believe that this new approach to oligonucleotide synthesis in solution will become the method of choice for the large scale preparation at least of oligonucleotides and oligonucleotide phosphorothioates of moderate chain length.

Acknowledgement

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13. A number of years ago we introduced[14] a system of abbreviations for protected oligonucleotides in which nucleoside residues and internucleotide linkages are italicized if they are protected in some defined way. In the present context, *A*, *C*, *G* and T represent 2'-deoxyadenosine protected on *N*-6 with a benzoyl group (as in **14**), 2'-deoxycytidine protected on *N*-4 with a benzoyl group (as in **15**), 2'-deoxyguanosine protected on *N*-2 and on *O*-6 with isobutyryl and 2,5-dichlorophenyl groups (as in **16**) and unprotected thymine, respectively; as indicated in Scheme 3, *p(s)* and *p(s')* represent *S*-(2-cyanoethyl) and *S*-(4-chlorophenyl) phosphorothioates, respectively, and p(H), which is not protected and therefore not italicized, represents an *H*-phosphonate monoester if it is placed at the end of a sequence or attached to a monomer (as in **8**, B = **14**), but otherwise it represents an *H*-phosphonate diester.
14. Di-(2-chlorophenyl) phosphorochloridate[15] (b.p. 172-175°C/1.0 mmHg, δ_P [$CDCl_3$] -5.0) was prepared in 78% yield by heating phosphoryl chloride with 2.0 mol. equiv. of 2-chlorophenol; it is a slightly superior coupling agent to diphenyl phosphorochloridate[16].
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19. *N*-[(4-Chlorophenyl)sulfanyl]phthalimide **19** (2.32 g, 8.0 mmol) was added with stirring to the solution of putative DMTr-*A*p(H)*C*-Lev[17], maintained at - 40°C. After 15 min, the products were worked up and chromatographed on silica gel.
20. van Boom and his coworkers[21] had previously reported that when the fully-protected dinucleoside *H*-phosphonate DMTr-Tp(H)T-Ac was treated with a very slight excess of *N*-(phenylsulfanyl)succinimide in the presence of diisopropylethylamine in dichloromethane solution, the corresponding *S*-phenyl phosphorothioate was obtained in 91% isolated yield.
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28. Under these reaction conditions, 'detritylation' is complete after 5 min. When 6-*N*-benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyadenosine was treated with hydrogen chloride in dioxane - dichloromethane under the same conditions, 'detritylation' was complete after 2 min, but no depurination could be detected even after 4 h.
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30. The oximate treatment step can be omitted if the oligonucleotide phosphorothioate does not contain any 2'-deoxyguanosine residues.
31. In each coupling step, a *ca.* 20% excess of *H*-phosphonate monomer **8** was used, but the excess of coupling agent **18** depended on the scale of the reaction; a twofold excess of sulfur-transfer agent **19** or **21** was used. The products were chromatographed on silica gel after each 'detritylation' step.
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