Diels-Alder cycloadditions in water for the straightforward preparation of peptide–oligonucleotide conjugates

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ABSTRACT

The Diels-Alder reaction between diene-modified oligonucleotides and maleimide-derivatized peptides afforded peptide–oligonucleotide conjugates with high purity and yield. Synthesis of the reagents was easily accomplished by on-column derivatization of the corresponding peptides and oligonucleotides. The cycloaddition reaction was carried out in mild conditions, in aqueous solution at 37°C. The speed of the reaction was found to vary depending on the size of the reagents, but it can be completed in 8–10 h by reacting the diene-oligonucleotide with a small excess of maleimide-peptide.

INTRODUCTION

In the past three decades, chemists involved in the preparation of synthetic oligonucleotide analogs suitable for use in the control of gene expression have introduced modifications in virtually every part of oligonucleotide chains [see, for instance (1–3)].

Among such modifications, the covalent attachment of peptides to oligonucleotides has received considerable attention because of the potential applications of peptide–oligonucleotide conjugates, such as the development of more effective oligonucleotide-based technologies. Peptides have been covalently linked to oligonucleotide chains for several purposes: with the aim of preparing nucleases (4–6), for the introduction of reporter groups (7), for the study of DNA–protein interactions (8), to investigate the molecular requirements for enzyme activity (9), to evaluate how metals or metal-based drugs behave when DNA and proteins are in close proximity (10,11), to increase the specificity of RNA-interacting oligonucleotides (12) and to facilitate the transport of antisense oligonucleotides through cell membranes (13–17). Linking peptides to oligonucleotides has also been described as rendering oligonucleotides more resistant to exonucleases (18–20), and, in the case of cationic peptides, as accelerating duplex formation (21). Positively charged and hydrophobic peptides stabilize short duplexes (22,23), but their influence on the thermal stability of duplexes with more than 15 bases is much weaker (12,24). Triplex stabilization by appending cationic peptides has also been described (25).

Since both peptides and oligonucleotides are biomolecules extremely rich in functional groups, finding reaction conditions that yield structurally defined conjugates rather than mixtures of products is far from being trivial. This problem has been tackled either by protecting non-participating functional groups, or by modifying the biomolecules with additional functional groups expected to drive the reaction as desired (26,27) [see also references (28–30) for examples of recent papers on the development of methodology for the synthesis of peptide–oligonucleotide conjugates].

The Diels-Alder reaction is a very attractive methodology for the conjugation of biomolecules, since it is fast and efficient in aqueous media (31–33) in addition to being chemoselective. It has been used, for instance, for the modification of peptides (34), to link covalently carbohydrates to proteins (35), for the immobilization of oligonucleotides on glass surfaces (36), and, most often, for the labelling of DNA and RNA fragments with biotin or fluoresceine derivatives (37–42). The Diels-Alder approach, which involves a diene and a dienophile not present in any biomolecule, allows for a chemoselective reaction without the need for protecting groups. Here, we describe the use of the Diels-Alder cycloaddition in water for the preparation of peptide–oligonucleotide conjugates incorporating all the nucleobases and most trifunctional amino acids. These conjugates were obtained by the reaction between an acyclic diene linked to the oligonucleotide chain and a maleimide-derivatized peptide (Figure 1).

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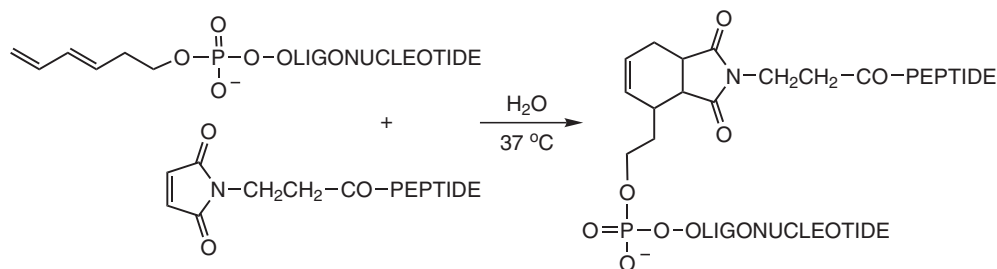
Figure 1. General scheme for the preparation of peptide–oligonucleotide conjugates by Diels-Alder reaction in water.

MATERIALS AND METHODS

N^{α}-Fmoc-L-amino acids and resins for peptide synthesis were obtained from Novabiochem (Fmoc = 9-fluorenylmethoxy-carbonyl). Trifunctional Fmoc-amino acid derivatives used: Arg(Pbf), Asn(Trt), Asp(OtBu), Gln(Trt), Glu(OtBu), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu) and Tyr(tBu) (Pbf = 2,2,4, 6,7-pentamethyldihydrobenzofuran-5-sulfonyl, Trt = trityl, tBu = *tert*-butyl, Boc = *tert*-butoxycarbonyl). The 3′-O-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite derivatives of DMT-dA^{Bz}, DMT-dC^{Bz}, DMT-dG^{iBu} and DMT-T, and 5′-DMT-nucleoside-LCAA-CPG (DMT = 4,4′-dimethoxytrityl, Bz = benzoyl, iBu = isobutyryl, LCAA-CPG = long chain aminoalkyl controlled pore glass beads) were purchased from the Glen Research Corporation. CH_2=CH-CH=CH-CH_2-CH_2-O-P(OCNE)NiPr$_2$ (CNE = 2-cyanoethyl) was prepared by phosphitylation of 3,5-hexadien-1-ol (43) as described previously (40). 3-Maleimidepropanoic acid was from Aldrich. Solid-phase syntheses were performed manually in a polypropylene syringe fitted with a polyethylene disc (peptides), or using the Expedite automatic DNA synthesizer from Applied Biosystems (oligonucleotides).

Reversed-phase high-performance liquid chromatography (HPLC) analyses were carried out on Kromasil C18 columns, using linear gradients of 0.045% TFA in H_2O and 0.036% TFA in ACN for peptide analyses (detection wavelength: 220 nm) (TFA = trifluoroacetic acid, ACN = acetonitrile), and 0.01 or 0.05 M aq. ammonium acetate and ACN/H_2O (1:1) for oligonucleotide analyses (detection wavelength: 260 nm). Purification by HPLC was carried out either using the same analytical Kromasil column (1 ml/min) or a PRP (Hamilton) column (2 ml/min).

A [Vydac C18]-filled glass column (22 × 2 cm) was used for medium pressure liquid chromatography (MPLC), using aqueous and ACN solutions containing 0.1% TFA in peptide purification, and 0.05 M ammonium acetate and 1:1 ACN/H_2O mixtures in oligonucleotide purification. Elution was carried out by connecting a piston pump to the mixing chamber of a gradient-forming device and to the top of the glass column. The mixing chamber of the gradient-forming device was the flask containing solvent A, which was connected through a stopcock to the flask containing solvent B.

Sephadex G-25 was used for gel filtration (elution with 0.05 M aq. ammonium acetate).

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometric analysis was carried out using a Voyager-DERP (Applied Biosystems) instrument and the following conditions: oligonucleotides and conjugates: trihydroxyacetophenone/ammonium citrate, negative mode, linear (unless otherwise indicated); peptides: 2,5-dihydroxybenzoic acid, positive mode, reflector. Calculated monoisotopic mass values for neutral compounds are indicated in all cases. The exact mass spectrometric characterization data were obtained using an Agilent 1100 LC/MS-TOF. Electrospray mass spectrometric analysis was carried out using a Micromass ZQ instrument.

The amounts of isolated oligonucleotides and conjugates were determined spectrophotometrically, and peptides were quantified by amino acid analysis after acid hydrolysis (6 M HCl for 1.5 h at 160°C).

Solution synthesis of diene-TT

T-3′-O-Pac. To a solution of 2.0 g of 5′-O-(4,4′-dimethoxy-trityl)-2′-deoxythymidine (3.7 mmol) and 2.8 g of phenoxy-acetic anhydride (9.8 mmol) in 50 ml of anh. THF was added 1.5 ml of pyridine (18.6 mmol), and the mixture was stirred overnight (Pac = phenoxyacetyl, THF = tetrahydrofuran). Removal of the solvent at reduced pressure afforded a yellow-ish oil, which was dissolved in DCM (75 ml) and extracted twice with a 10% aq. NaHCO$_3$ solution (100 ml, 2×) (DCM = dichloromethane). The aqueous phase was re-extracted with 50 ml of DCM, and the combined organic phases were dried over anh. Na$_2$SO$_4$, filtered and evaporated to dryness. The resulting oil was redissolved in the minimum amount of AcOEt and precipitated with cold hexanes. After centrifugation, 5′-O-DMT-T-3′-O-Pac was obtained as a white solid (1.82 g, 73% yield). The fully protected thymidine derivative (1.82 g, 2.7 mmol) was dissolved in an 8:2 DCM/MeOH solution, and cooled in an ice bath. A solution of 2.28 g of *p*-toluenesulfonic acid (12 mmol) in the same solvent was added, and the mixture was stirred for 1 h at 0°C. The reaction was quenched by addition of 100 ml of a 10% aq. NaHCO$_3$ solution. The aqueous phase was extracted with DCM (60 ml), and the combined organic phases were dried over anh. Na$_2$SO$_4$, filtered, and evaporated to dryness. The 3′-protected thymidine derivative was purified by silica gel column chromatography, eluting with DCM/AcOEt mixtures of increasing polarity, and obtained as a white solid (415 mg, 42% yield). Mp: 156–158°C; R_f (CH$_2$Cl$_2$): 0.37; ^{1}H-NMR (200 MHz, CDCl$_3$), δ (p.p.m.): 1.26 (s, 3H, CH$_3$), 2.40 (m, 2H, H$_{2'}$ and H$_{2''}$), 3.93 (m, 2H, H$_{5'}$ and H$_{5''}$), 4.11 (m, 1H, H$_{4'}$), 4.69 (s, 2H, CH$_2$(Pac)), 5.50 (d, 1H, H$_{3'}$, J = 8 Hz), 6.20 (t, 1H, H$_{1'}$, J = 7.1 Hz), 6.90–7.30 (m, 5H, Ph), 7.50 (s, 1H, H$_6$), 8.60 (s, 1H, NH); ES-MS, positive mode: m/z 399.1163 [M+Na]$^+$ (calcd mass for C$_{18}$H$_{20}$N$_2$O$_7$Na: 376.1162, error: 0.069 p.p.m.).

T-P(O)(OCNE)-T-3′-O-Pac. 273 mg of T-3′-O-Pac (0.73 mmol) and 115 mg of sublimed tetrazole (1.64 mmol) were coevaporated from anh. ACN (3×) and dissolved in 3 ml of anh. ACN. To this solution was added a solution of 5′-O-DMT-T-3′-O-P(OCNE)NiPr$_2$ (1 g, 1.34 mmol) in 6 ml anh. ACN with a cannula, and the mixture was stirred for 1.5 h. A total of 1.5 ml of a 6.0 M solution of *t*BuOOH in decane was added (9.0 mmol), and after 30 min stirring, the mixture was diluted with 50 ml of DCM and extracted with a 2.5% aq. NaHCO$_3$ solution (50 ml, 2×) and brine (50 ml). The organic solution was dried over anh. Na$_2$SO$_4$, filtered, and evaporated to dryness, which afforded a yellowish oil. The DMT group was removed from the fully protected dimer as described above, and T-P(O)(OCNE)-T-3′-O-Pac was obtained as a brownish solid after silica gel column chromatography eluting with DCM and increasing amounts of MeOH (1–8%) (115 mg, 22% yield). R$_f$ (CH$_2$Cl$_2$/MeOH 93:7): 0.38; ^{1}H-NMR (400 MHz, CDCl$_3$), δ (p.p.m.): 1.92 (s, 3H, CH$_3$), 1.96 (s, 3H, CH$_3$), 2.44 (m, 2H, H$_{2'}$ and H$_{2''}$), 2.45 (m, 2H, H$_{2'}$ and H$_{2''}$), 2.79 (t, 2H, CH$_2$-CH$_2$-CN, J = 5.6 Hz), 3.88 (s, 2H, H$_{5'}$ and H$_{5''}$), 4.19 (s, 2H, H$_{5'}$ and H$_{5''}$), 4.24 (t, 2H, CH$_2$-CH$_2$-CN, J = 5.6 Hz), 4.32 (m, 1H, H$_{4'}$), 4.37 (m, 1H, H$_{4'}$), 4.71 (s, 2H, CH$_2$, phenoxyacetyl), 5.20 (m, 1H, H$_{3'}$), 5.50 (m, 1H, H$_{3'}$), 6.16 (t, 1H, H$_{1'}$, J = 7 Hz), 6.22 (t, 1H, H$_{1'}$, J = 7 Hz), 6.90–7.34 (m, 5H, Ph), 7.39 (s, 1H, H$_6$), 7.40 (s, 1H, H$_6$), 8.00–8.15 (broad signal, NH); ^{31}P-NMR (101,2 MHz, CDCl$_3$), δ (p.p.m.): −3.38, −3.49; ES-MS, positive mode: *m/z* 734.2070 [M+H]$^+$ (calcd mass: for C$_{31}$H$_{37}$N$_5$O$_{14}$P: 734.2069, error: 0.114 p.p.m.).

CH$_2$=CH-CH=CH-CH$_2$-CH$_2$-O-p-T-p-T (diene-TT). The reaction between CH$_2$=CH-CH=CH-CH$_2$-CH$_2$-O-P(OCNE)NiPr$_2$ (160 mg, 0.54 mmol) and T-P(O)(OCNE)-T-3′-O-Pac (115 mg, 0.16 mmol) was carried out as described above for the preparation of the protected TT-dimer using 3.4 equivalent of diene-phosphoramidite with respect to the TT-dimer. After the aqueous work-up, removal of the 2-cyanoethyl and phenoxyacetyl protecting groups was accomplished by reaction with 8 ml of a conc. aq. ammonia solution (32%) for 3 h at room temperature. After evaporation to dryness, the target diene-TT dimer was purified by MPLC eluting with a gradient from 0 to 100% of B (A: 0.05 M NH$_4$AcO, B: [70% 0.05 M NH$_4$AcO + 30% (ACN/H$_2$O 1:1, v/v)], 600 ml of each solvent). After lyophilization, 47 mg of a slightly yellow solid, pure as shown by HPLC analysis, were obtained (43% yield). MALDI-TOF MS (reflector): *m/z* 704.78 [M-H]$^-$ (calcd mass: 704.15); ES-MS, positive mode: *m/z* 707.1723 [M+H]$^+$ (calcd mass: for C$_{26}$H$_{37}$N$_4$O$_{15}$P$_2$: 707.1725, error: 0.312 p.p.m.); anal. HPLC: linear gradient from 5 to 40% of B in 30 min, t_R 18.1 min.

Solid-phase synthesis of diene-oligonucleotides

Oligonucleotides were assembled on CPG at the 1 μmol-scale following standard procedures (phosphite triester approach). No changes in the synthesis cycle were made for the incorporation of the diene-phosphoramidite. Final deprotection was carried out by reaction with conc. aqueous ammonia, either overnight at 55°C (diene derivatives of oligodeoxynucleotides CATGGCT and GATCTAAAAGACTTT) or for 4 h at room temperature (T$_8$ and T$_{15}$ derivatives).

Diene-CATGGCT. Purification was accomplished by MPLC eluting with a gradient from 0 to 100% of B (A: 0.05 M NH$_4$AcO, B: [70% 0.05 M NH$_4$AcO + 30% (ACN/H$_2$O 1:1, v/v)], 600 ml of each solvent). A total of 34% synthesis and purification yield; MALDI-TOF MS: *m/z* 2254.98 [M-H]$^-$ (calcd mass: 2255.80); anal. HPLC: linear gradient from 5 to 40% of B in 30 min, t_R 17.0 min.

Diene-GATCTAAAAGACTTT. Purification was accomplished by MPLC eluting with a gradient from 0 to 100% of B (A: 0.05 M NH$_4$AcO, B: [70% 0.05 M NH$_4$AcO + 30% (ACN/H$_2$O 1:1, v/v)], 600 ml of each solvent). A total of 32% synthesis and purification yield; MALDI-TOF MS: *m/z* 4725.20 [M-H]$^-$ (calcd mass: 4732.70); anal. HPLC: linear gradient from 5 to 60% of B in 30 min, t_R 13.9 min.

Diene-TTTTTTTT. Purification was accomplished by MPLC eluting with a gradient from 0 to 100% of B {A: [90% 0.05 M NH$_4$AcO + 10% (ACN/H$_2$O 1:1, v/v)], B: [65% 0.05 M NH$_4$AcO + 35% (ACN/H$_2$O 1:1, v/v)], 600 ml of each solvent}. A total of 39% synthesis and purification yield; MALDI-TOF MS: *m/z* 2530.56 [M-H]$^-$ (calcd mass: 2530.44); anal. HPLC: linear gradient from 10 to 40% of B in 30 min, t_R 18.4 min.

Diene-TTTTTTTTTTTTTTT. Purification was accomplished by MPLC eluting with a gradient from 0 to 100% of B (A: [90% 0.05 M NH$_4$AcO + 10% (ACN/H$_2$O 1:1, v/v)], B: [65% 0.05 M NH$_4$AcO + 35% (ACN/H$_2$O 1:1, v/v)], 600 ml of each solvent). A total of 21% synthesis and purification yield; MALDI-TOF MS: *m/z* 4652.88 (calcd mass: 4658.76), electrospray MS: *m/z* 4661.18 [M-H]$^-$ (calcd average mass: 4661.04); anal. HPLC: linear gradient from 10 to 40% of B in 30 min, t_R 18.0 min.

Synthesis of maleimide-peptide-NH$_2$

All the amino acids of the peptide sequence were subsequently coupled on a Rink amide *p*-methylbenzhydrylamine resin (44) (100 mg, loading: 69 μmol NH$_2$/mg) following the standard procedures of solid-phase peptide synthesis (10 min treatment with 20% piperidine in *N,N*-dimethylformamide and reaction with 3 equivalent of Fmoc-amino acid and DCC for 1–1.5 h were used for the deprotection and coupling steps, respectively), with the addition of 3 equivalent of HOBt for the incorporation of 3-maleimidepropanoic acid, and in all couplings in the octapeptide assembly (DCC = *N,N′*-dicyclohexylcarbodiimide, HOBt = 1-hydroxybenzotriazole). Cleavage and deprotection were effected by reaction with TFA/H$_2$O 95:5 in the case of the dipeptides (30 min), and with a TFA/H$_2$O/TIS 90:5:5 mixture (5 h) in the case of the octapeptide (TIS = triisopropylsilane). Most of the TFA was removed by bubbling N$_2$ into the solution, and the resulting residue was either diluted with H$_2$O and lyophilized (dipeptides) or poured onto cold ether to precipitate the target molecule (octapeptide). Crude maleimide-octapeptide was obtained after centrifugation and solvent removal. All maleimide-peptides were purified by MPLC eluting with a gradient from 0 to 100% of B (A: 0.1% TFA in H$_2$O, B: [60% (0.1% TFA in H$_2$O) + 40% (0.1% TFA in ACN)], 600 ml of each solvent).

Maleimide-Ala-Gly-NH$_2$. A total of 27% synthesis and purification yield; MALDI-TOF MS: *m/z* 297.08 [M+H]$^+$

(calcd mass: 296.43); amino acid composition: Gly 0.95, Ala 1.05; anal. HPLC: linear gradient from 0 to 50% of B in 30 min, t_R 10.1 min.

Maleimide-Asp-Gly-NH$_2$. A total of 9% synthesis and purification yield; MALDI-TOF MS: m/z 340.91 [M+H]$^+$ (calcd mass: 340.41); amino acid composition: Asp 0.87, Gly 1.12; anal. HPLC: linear gradient from 0 to 50% of B in 30 min, t_R 9.7 min.

Maleimide-Lys-Gly-NH$_2$. A total of 41% synthesis and purification yield; MALDI-TOF MS: m/z 354.26 [M+H]$^+$ (calcd mass: 353.58); amino acid composition: Gly 1.10, Lys 0.90; anal. HPLC: linear gradient from 0 to 50% of B in 30 min, t_R 9.5 min.

Maleimide-Ser-Gly-NH$_2$. A total of 42% synthesis and purification yield; MALDI-TOF MS: m/z 312.96 [M+H]$^+$ (calcd mass: 312.42); amino acid composition: Ser 0.87, Gly 0.95; anal. HPLC: linear gradient from 0 to 50% of B in 30 min, t_R 9.1 min.

Maleimide-Gly-Thr-Ser-Lys-Leu-Asn-Tyr-Leu-NH$_2$. A total of 22% synthesis and purification yield; MALDI-TOF MS: m/z 1044.95 [M+H]$^+$ (calcd mass: 1046.15); amino acid composition: Asp 0.96, Thr 0.75, Ser 0.99, Gly 1.22, Leu 2.32, Tyr 0.81, Lys 0.95; anal. HPLC: linear gradient from 0 to 50% of B in 30 min, t_R 20.8 min.

Synthesis of maleimide-peptide-OH

The peptide was assembled on a 2-chlorotrityl chloride resin (45) (360 mg, loading: 1.49 μmol Cl/mg). To prepare the Fmoc-Ala-resin, the solid matrix was treated with a mixture of Fmoc-Ala-OH (0.6 equivalent 100.2 mg) and *N*-ethyldiiso-propylamine (5 equivalent 456 μl) for 45 min, and the remaining reactive groups were capped by reaction with 400 μl of methanol. Deprotection of an aliquot of Fmoc-Ala-resin and quantification of the resulting 9-fluorenylmethylpiperidine showed that the loading was 0.63 μmol Fmoc/mg. The remaining amino acids were incorporated using standard solid-phase procedures (DIPC/HOBt-mediated couplings, DIPC = *N*,*N*′-diisopropylcarbodiimide). The peptide-resin bond was cleaved by reaction with a TFA/EDT/DCM (EDT = 1,2-ethane-dithiol) 1:1:98 mixture (5 × 30 s treatment), and amino acid side chains were deprotected by adding a 2.5:2.5:2.5:1:91.5 thioanisole/H$_2$O/EDT/TIS/TFA mixture to the resulting fil-trate (4.5 h, r. t.). Most of the TFA was removed by bubbling N$_2$ into the solution, and the resulting residue was poured onto cold ether to precipitate the target maleimide-peptide. Purification was accomplished by MPLC eluting with a gradient from 0 to 100% of B (A: [85% (0.1% TFA in H$_2$O) + 15% (0.1% TFA in ACN)], B: [55% (0.1% TFA in a total of H$_2$O) + 45% (0.1% TFA in ACN)], 600 ml of each solvent).

Maleimide-Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala-OH: 25% yield; MALDI-TOF MS: m/z 2316.98 [M+H]$^+$ (calcd mass: 2316.04); amino acid analysis after acid hydrolysis: Asp 1.07, Thr 1.95, Ser 2.84, Glu 3.15, Ala 4.97, Met 0.97, Phe 1.08, His 1.15, Lys 1.97, Arg 1.15; anal. HPLC: linear gradient from 10 to 40% of B in 30 min, t_R 16.7 min.

Diels-Alder conjugation reactions

Reaction between diene-TT and maleimide-dipeptides. Diene-TT and each of the maleimide-peptides were dissolved in water and mixed to give 1 mM solutions containing a 1:1 molar ratio of the reagents (35 nmol). The reaction mixtures were stirred at 37°C, and the progress of the reaction was monitored by HPLC. A total of 90–95% conjugate was formed in reaction times ranging between 4 and 14 h, as assessed from the relative areas of the diene-dinucleotide and the conjugate on the HPLC profile. Two main products, corresponding to the two diastereomeric forms of the target conjugate, were formed in all cases, and showed different retention times upon HPLC analysis (Figure 2A). For MALDI-TOF MS characterization (products isolated by HPLC), see Table 1.

Anal. HPLC: linear gradient from 5 to 40% of B in 30 min, t_R: 15.1 and 15.5 min (TT-Ala-Gly conjugate), 12.5 and 12.9 min (TT-Asp-Gly conjugate), 15.6 and 16.0 (TT-Lys-Gly con-jugate), 14.5 and 15.0 (TT-Ser-Gly conjugate).

The reaction between diene-TT and maleimide-Ser-Gly-NH$_2$ was repeated at the μmol-scale (2.9 μmol diene + 4.3 μmol dienophile, peptide/oligonucleotide molar ratio 1:1.5, diene-oligonucleotide concentration in the reaction medium: 1.9 mM, maleimide-peptide concentration: 2.8 mM, 24 h), and the conjugate was isolated by HPLC eluting with a gradient from 5 to 45% of B in 30 min (A: 0.01 M NH$_4$AcO, B: ACN/H$_2$O 1:1, Kromasil C18 column). Conjugation and purification yield: 60%.

Reaction between maleimide-GTSKLNYL-NH$_2$ and larger diene-oligonucleotides. The cycloaddition reaction was also carried out in water at 37°C, and monitored by HPLC and MALDI-TOF MS. The molar ratio and reaction time were adjusted in each case depending on the kinetics of the process. The two conjugates were purified by HPLC and characterized by mass spectrometry (Table 1).

Maleimide-GTSKLNYL-NH$_2$ + diene-CATGGCT. 5.7 nmol maleimide-peptide + 2.8 nmol diene-oligonucleotide, 2:1

Table 1. Mass spectrometric characterization data of the synthesized conjugates

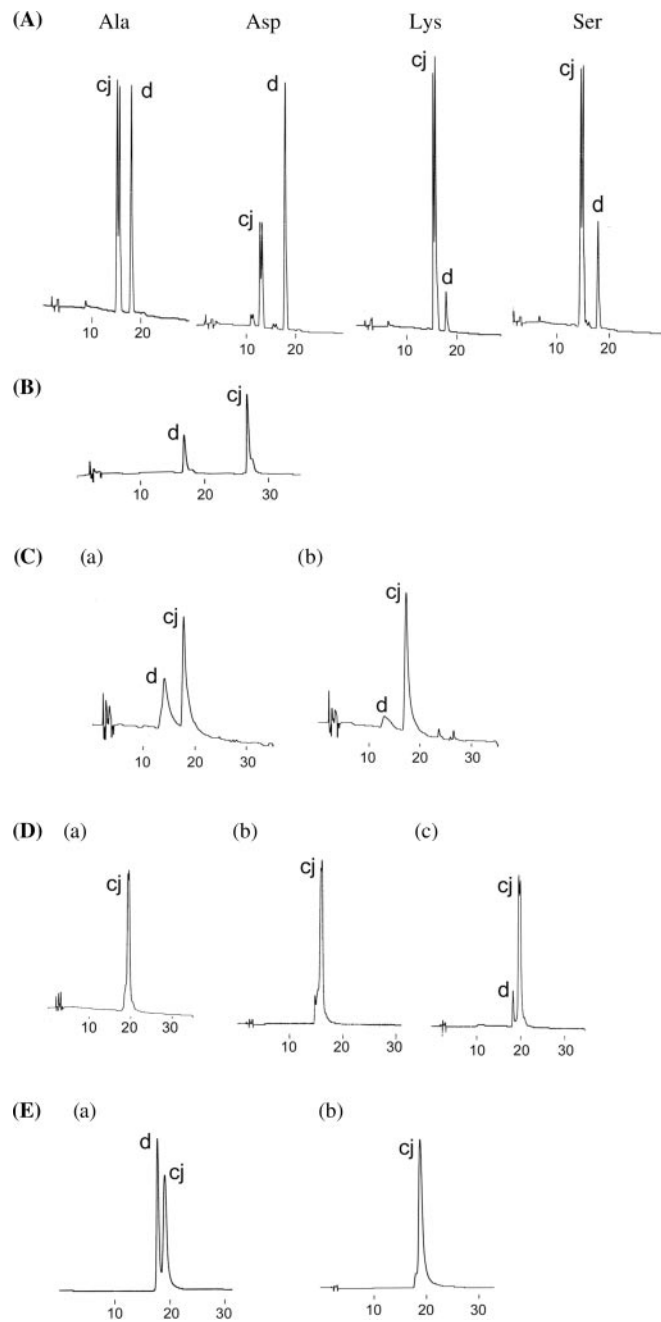| Peptide	Oligonucleotide	Conjugate: MALDI-TOF MS Found [M-H]$^-$	Calcd M
AG-NH$_2$	TT	1000.10	1000.58
KG-NH$_2$	TT	1045.43	1044.56
SG-NH$_2$	TT	1057.02	1057.63
DG-NH$_2$	TT	1015.92	1016.57
GTSKLNYL-NH$_2$	CATGGCT	3297.97	3301.13
GTSKLNYL-NH$_2$	GATCTAAAAGACTTT	5776.62	5778.85
KETAAAKFERQHMDSSTSAA-OH	TTTTTTTT	4842.21	4847.89
KETAAAKFERQHMDSSTSAA-OH	TTTTTTTTTTTTTTTT	6968.32	6974.80

Figure 2. Reversed-phase HPLC traces of the conjugation reactions (d, diene-oligonucleotide; cj, conjugate). (**A**) maleimide-dipeptide-NH_2 + diene-TT 1:1 after 4 h (the *N*-terminal amino acid of the dipeptide is indicated on top of each profile); (**B**) maleimide-GTSKLNYL-NH_2 + diene-CATGGCT 2:1 after 2.5 h; (**C**) maleimide-GTSKLNYL-NH_2 + diene-GATCTAAAAGACTTT, 2:1 after 6 h (a) and 5:1 after 1 h (b); (**D**) maleimide-KETAAAKFERQHMDSSTSAA-OH + diene-TTTTTTTT 1:1 after 20 h, first (a), second (b) and third (c) assay (see Materials and Methods); (**E**) maleimide-KETAAAKFERQHMDSST-SAA-OH + diene-TTTTTTTTTTTTTTT 1:1 after 20 h (a), and 8 h later after having added the amount of peptide equivalent to that of unreacted oligonucleotide (b).

ratio, diene-oligonucleotide concentration in the reaction medium: 0.05 mM, 70% conjugation yield in 2.5 h (HPLC analysis, Figure 2B). Anal. HPLC: linear gradient from 5 to 40% of B in 30 min, t_R 26.9 min.

Maleimide-GTSKLNYL-NH_2 + diene-GATCTAAAAGA-CTTT. 3.0 nmol maleimide-peptide + 1.5 nmol diene-oligonucleotide, 2:1 ratio, diene-oligonucleotide concentration in the reaction medium: 0.05 mM, 72% conjugation yield in 6 h [HPLC analysis, Figure 2C, (a)]; 7.7 nmol maleimide-peptide + 1.5 nmol diene-oligonucleotide, 5:1 ratio, diene-oligonucleotide concentration in the reaction medium: 0.05 mM, >95% conjugation yield in 1 h [HPLC analysis, Figure 2C, (b)]. Anal. HPLC: linear gradient from 5 to 60% of B in 30 min, t_R 17.6 min.

Maleimide-KETAAAKFERQHMDSSTSAA-OH + diene-T_8. The cycloaddition reaction between equimolar amounts of the two reagents (55 nmol, 0.03 mM) was carried out in water at 37°C, and monitored by HPLC and MALDI-TOF MS: 80% conjugation yield in 4 h, >95% conjugation yield after 20 h [HPLC analysis, Figure 2D, (a)].

The reaction between maleimide-KETAAAKFERQHMD-SSTSAA-OH and diene-T_8 was repeated two more times at a higher scale, using 1:1 molar ratios of peptide and oligonucleotide, and the mixture analyzed by HPLC only after 20 h. Since the conversion of diene-oligonucleotide to peptide–oligonucleotide conjugate after that time was not quantitative, an amount of maleimide-peptide equimolar to the amount of unreacted diene-oligonucleotide was added, and the mixture was left to react for a further 4 h. The reaction mixture was lyophilized, and the target conjugate was isolated, in all cases, after gel filtration through Sephadex G-25. The following results were obtained:

Second assay: 156 nmol-scale, 0.08 mM, 94% conjugation yield after 20 h [HPLC analysis, Figure 2D, (b)]. Purification: gel filtration. Conjugation and purification yield: 59%.

Third assay: 110 nmol-scale, 0.05 mM, 90% conjugation yield after 20 h [HPLC analysis, Figure 2D, (c)]. Purification: gel filtration. Conjugation and purification yield: 65%.

Anal. HPLC: linear gradient from 10 to 40% of B in 30 min, t_R 19.7 min. For mass spectrometric characterization, see Table 1.

Maleimide-KETAAAKFERQHMDSSTSAA-OH + diene-T_{15}. Equimolar amounts (190 nmol) of the two reagents were mixed in water (0.18 mM) and stirred at 37°C. The conjugation yield after 8 h was 56% [HPLC analysis, Figure 2E, (a)]. An amount of maleimide-peptide equimolar to the amount of unreacted diene-oligonucleotide was added, and the mixture was left to react for a further 8 h [Figure 2E, (b)]. The target conjugate was obtained after purification by gel filtration. Some fractions required additional repurification to remove small amounts of accompanying diene-oligonucleotide (HPLC, PRP column, linear gradient from 15 to 35% of B in 30 min, A: 0.05 M NH_4AcO, B: ACN/H_2O 1:1, 2 ml/min). Conjugation and purification yield: 62%. Anal. HPLC: linear gradient from 10 to 40% of B in 30 min, t_R 19.3 min. For mass spectrometric characterization, see Table 1.

RESULTS AND DISCUSSION

As stated above, the goal of this study was to prepare peptide–oligonucleotide conjugates using the Diels-Alder cycloaddition. The target conjugates were easily obtained by reaction between diene-oligonucleotides and maleimide-peptides. This
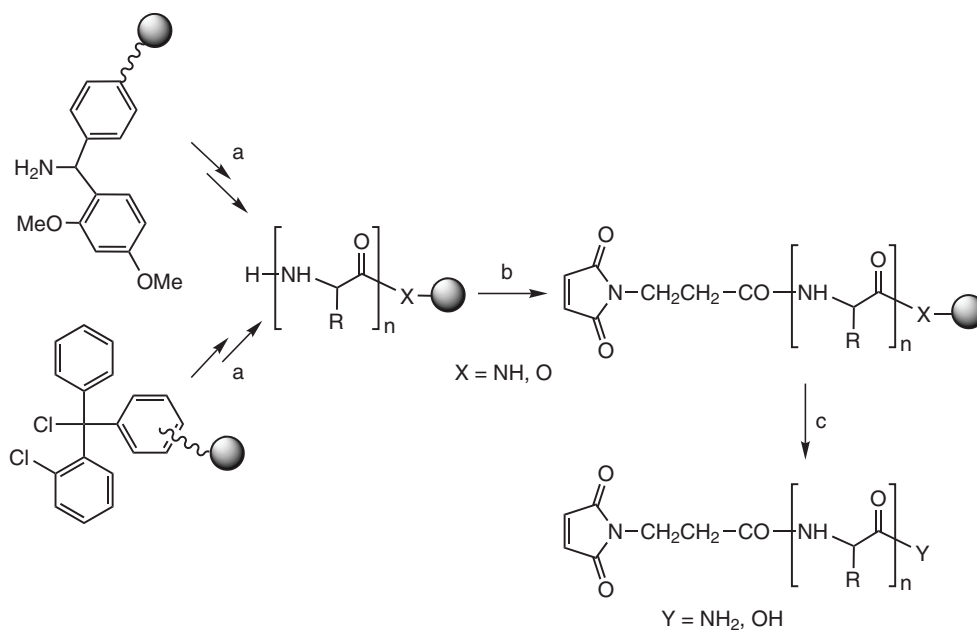
Figure 3. Preparation of the maleimide-peptides: (a) assembly of the peptide chain using Fmoc-amino acids on Rink amide (top) or 2-chlorotrityl chloride (bottom) resins; (b) coupling of 3-maleimidepropanoic acid in the presence of DCC (or DIPC) and HOBt; (c) final deprotection with a 90–95% TFA solution.

alternative is much simpler than synthesizing maleimide-oligonucleotides and diene-peptides, since the maleimide moiety is not stable to the ammonia treatment used for the final deprotection of oligonucleotides (46), and the diene would not resist the acidic conditions in which peptide permanent protecting groups are removed. In all cases, derivatization was carried out after chain assembly following standard protocols, thus yielding 5′-modified oligonucleotides and peptides with the maleimide group linked to the N-terminal.

Several peptides and oligonucleotides with different composition and length were synthesized for this study (Table 1). This allowed a variety of conjugates to be obtained, from dipeptide-dinucleotide conjugates to conjugates incorporating 20mer peptides and 15mer oligonucleotides.

Peptide chains were assembled by solid-phase synthesis using Fmoc/tBu-protected amino acid derivatives (47,48). The commercially available 3-maleimidepropionic acid was coupled onto the N-terminal of the immobilized peptide chains. Peptide elongation on a Rink amide resin (44) afforded maleimide-peptides with a C-terminal carboxamide, and the 2-chlorotrityl chloride resin (45) was used to obtain the maleimide-peptide-OH. Deprotection of the immobilized maleimide-peptides was carried out by treatment with TFA (Figure 3) in the presence of the appropriate scavengers. Maleimide-peptides were isolated by medium pressure liquid chromatography and characterized by MALDI-TOF MS and amino acid analysis.

The diene-derivatized TT dinucleotide was synthesized in solution at the milligram-scale (Figure 4) (see Materials and Methods for details), to obtain the amount of material required for the preliminary conjugation assays with the different maleimide-dipeptides. The other diene-oligonucleotides were assembled on controlled pore glass beads using standard phosphite triester methodology (49). After the subsequent incorporation of the different

nucleosides, the phosphoramidite derivative of 3,5-hexadiene-1-ol, previously prepared following described procedures (43,40), was coupled onto the 5′ end (Figure 5). Deprotection of diene-oligonucleotides was accomplished by treatment with conc. aq. ammonia, at room temperature for thymidine-containing chains, and at 55°C for oligonucleotides incorporating the four nucleobases. HPLC and mass spectrometric analysis of the diene-oligonucleotide crudes showed that no measurable side reactions took place upon incorporation of the phosphoramidite-derivatized 3,5-hexadien-1-ol onto the oligonucleotide chains, which indicates that the diene group remains stable to oxidation with either tBuOOH or aq. iodine. Diene-oligonucleotides were purified by medium pressure liquid chromatography. Characterizationon was carried out by MALDI-TOF mass spectrometric analysis, and purity was confirmed by reversed-phase HPLC. Although it is generally observed that longer oligonucleotide chains have higher retention times, the retention time of diene-TT was higher than that of diene-CATGGCT, and diene-T_8 and diene-T_{15} had virtually the same retention time (see Materials and Methods for details). This is probably related to the presence of the hydrophobic diene substituent, whose specific contribution to the chromatographic behavior of the molecule is higher in the shortest diene-modified oligonucleotides.

The Diels-Alder conjugation reaction was carried out simply by mixing aqueous solutions of the diene-modified oligonucleotide and maleimide-derivatized peptide, and stirring the resulting solution at 37°C. The progress of the reaction was monitored by reversed-phase HPLC (see Figure 2) and MALDI-TOF mass spectrometry (analysis in the positive mode may allow detection of maleimide–peptide in the reaction mixture, but the mass spectrometric analysis is usually conducted in the negative mode to allow detection of diene-oligonucleotides and conjugates). The target
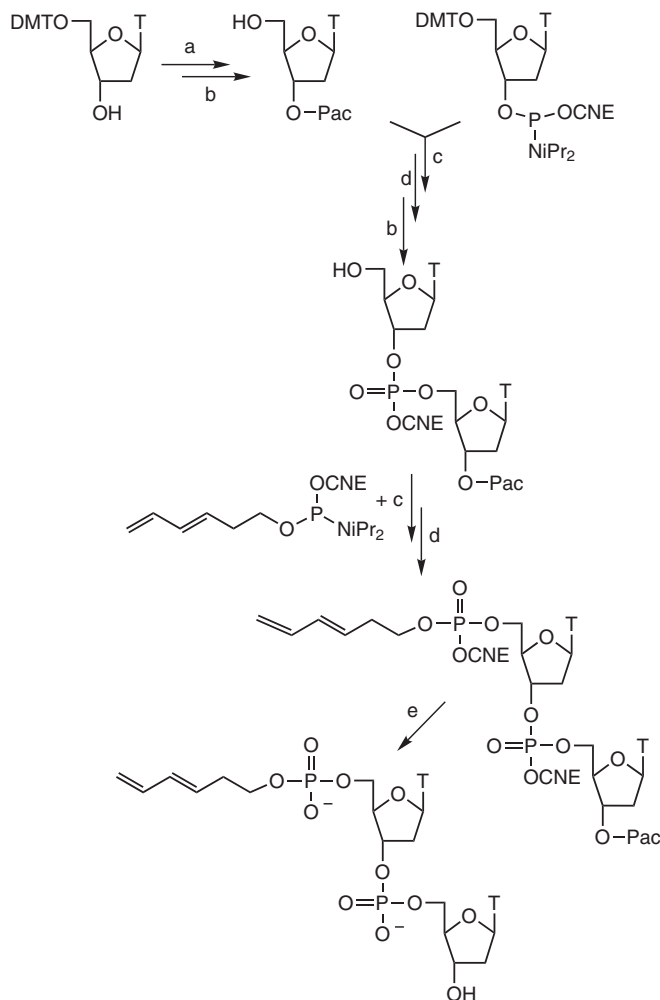
Figure 4. Solution synthesis of diene-TT: (a) (PhOCH$_2$CO)$_2$O; (b) *p*TsOH; (c) tetrazole; (d) *t*BuOOH; (e) conc. aq. NH$_3$.

peptide–oligonucleotide conjugates were formed in all cases and were easily isolated after gel filtration through Sephadex G-25 or reversed-phase HPLC. The conjugate structure was confirmed by MALDI-TOF mass spectrometric analysis (Table 1).

The first assays were carried out with the maleimide-dipeptides and diene-TT (1:1 molar ratio). The reaction was clean and fast in a few hours, as shown by HPLC analysis at different reaction times. The four crudes contained two main products (Figure 2A), which were isolated by HPLC and characterized by MALDI-TOF MS. In all cases, the two products had the same mass, that of a Diels-Alder adduct. Only the diastereomers of these small conjugates could be separated by HPLC, but not those of the larger ones (Figure 2B–E). We surmise that these products are the two diastereomeric conjugates resulting from the *endo* addition, which is usually preferred over the *exo*. Detailed structural analysis of the stereochemistry of these adducts is beyond the scope of this paper. Both diastereomeric products can be useful for biological applications.

It was also observed that the kinetics of the reaction depended on the nature of the peptide, the order of reactivity being KG>SG>AG>>DG (see HPLC traces after 4 h in

Figure 2A). These data indicate that the reaction rate increases when favourable interactions between the two moieties, such as those between the negatively charged oligonucleotide and a positively charged peptide, can be established. The polar hydroxyl group of the serine side chain also had a positive effect, and the slowest reaction rate was found when two negatively charged chains were brought together into the same conjugate.

The second set of experiments involved an octapeptide (maleimide-GTSKLNYL-NH$_2$) and two oligonucleotides containing the four nucleobases (diene-CATGGCT and diene-GATCTAAAAGACTTT). These conjugations were carried out using 2:1 peptide/oligonucleotide ratios. The HPLC monitoring of these experiments showed that, as expected, the rate of the reaction varied inversely with the size of the diene reagent. A total of 70% conjugation yield was achieved in 2.5 h when the diene was linked to the 7mer oligonucleotide, and in 6 h in the case of the 15mer oligonucleotide (Figure 2B and C). The reaction between maleimide-GTSKLNYL-NH$_2$ and diene-GATCTAAAAGACTTT was nearly quantitative in 1.5 h when a 5:1 peptide/oligonucleotide ratio was used.

The last group of experiments was performed with a 20mer peptide (maleimide-KETAAAKFERQHMDSSTSAA-OH) and two oligothymidines, diene-T$_8$ and diene-T$_{15}$. To evaluate whether large conjugates could be obtained in good yields with the lowest cost in reagents, the diene and the dienophile were reacted in 1:1 molar ratio. The HPLC analysis of these reactions showed that the conjugation process was not complete in 20–24 h, the reaction rate, again, showing an inverse dependence on the reagents' size (see details in Materials and Methods). One way to drive these reactions to completion was to increase the reaction time. However, it has been suggested that undesired reactions between the free amines of the amino acid side chains and the maleimide group of the peptide moiety may take place at prolonged reaction times (50). Moreover, although this side reaction is faster in basic conditions, some maleimide can be lost by hydrolysis (51). Therefore, extending the reaction time over a period of days did not seem the best choice, and we decided to add the required extra amount of fresh maleimide-peptide (equimolar amount with respect to unreacted oligonucleotide, as assessed by HPLC analysis). A further 4 h was enough to complete the conjugation process for the diene-T$_8$, and 8 h in the case of diene-T$_{15}$.

The reaction between the 20mer peptide and diene-T$_8$ was repeated three times. The HPLC analysis after 20 h showed that either the diene-oligonucleotide had been consumed, or that the crude contained some 5–10% of unreacted diene. As stated above, in all cases the reaction was completed in 4 h after the required amount of peptide was added. These results show the reproducibility of the method.

The cycloaddition reaction between two diene moieties was not detected in any case. This is as expected, since the energy of activation of this process is much higher, and hence this reaction is much slower than the cycloaddition between the diene and the maleimide groups.

In summary, the results presented here demonstrate the utility of the Diels-Alder reaction for the easy, side-reaction free preparation of peptide–oligonucleotide conjugates under mild conditions. Diene-oligonucleotides and maleimide-peptides can be easily prepared using standard solid-phase
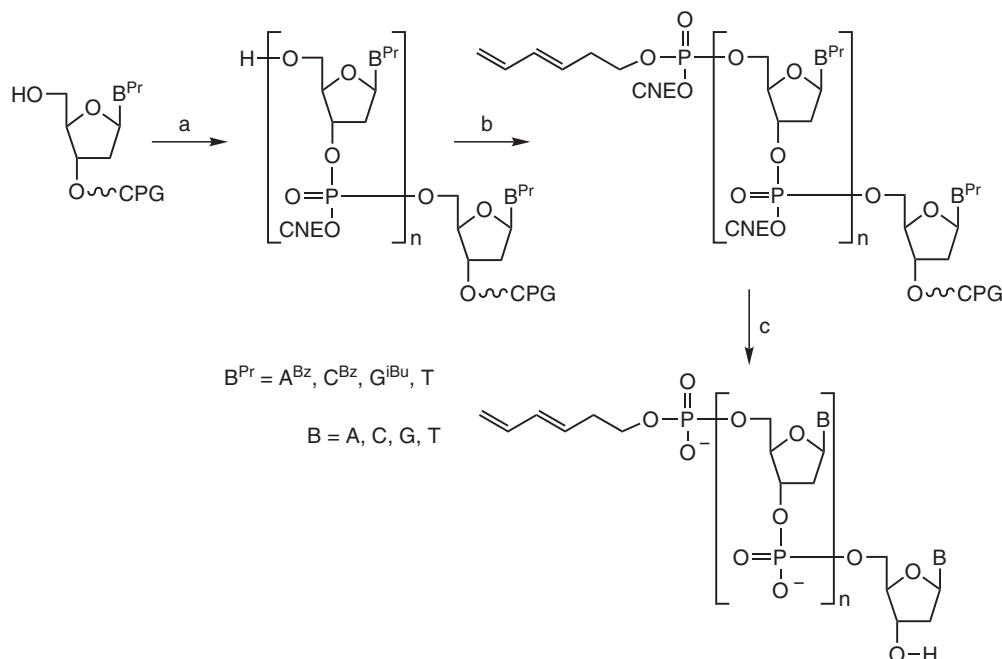
Figure 5. Solid-phase synthesis of diene-oligonucleotides: (a) oligonucleotide assembly using standard $3'$-phosphoramidite nucleoside derivatives; (b) tetrazole-mediated coupling of $CH_2=CH-CH=CH-CH_2-CH_2-O-P(OCNE)NiPr_2$, followed by capping and oxidation (aq. I_2); (c) final deprotection with conc. aq. ammonia.

methodologies. Cycloaddition reactions in water are clean, fast and chemoselective, and they allow the straightforward synthesis of large peptide–oligonucleotide conjugates, containing any nucleoside or trifunctional amino acid, which are otherwise difficult to obtain (26,27).

Certain adjustments in the methodology described here will be required if cysteine is to be included in the peptide sequence. The reaction between free thiols and maleimide groups, which has been exploited for the preparation of peptide–oligonucleotide conjugates (26), may compete with the desired Diels-Alder cycloaddition, yielding side products in which peptide chains are linked to each other as a result of Michael additions. Furthermore, the thiols of two peptide molecules may react to give disulfide-linked peptide dimers. To avert the production of complex mixtures during conjugation, we intend to carry out the cycloaddition using maleimide-peptides with a protected cysteine residue, and to unmask the thiol group after the conjugation has taken place.

Work is in progress to extend the use of the Diels-Alder reaction for the preparation of different types of bioconjugates, including cysteine-containing peptide–oligonucleotide hybrids.

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