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Synthesis and binding properties of conjugates between oligodeoxynucleotides and daunorubicin derivatives

Anna Garbesi*, Stefania Bonazzi, Stefania Zanella, Massimo L. Capobianco, Giuseppe Giannini[1] and Federico Arcamone[1]

I.Co.CEA–CNR, Via P.Gobetti 101, 40129 Bologna, Italy and [1]Menarini Ricerche, Via T.Speri 10, 00040 Pomezia, Roma, Italy

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ABSTRACT

Conjugation of an anthracycline to a triplex-forming oligonucleotide (TFO) allows delivery of this drug to a specific DNA site, preserving the intercalation geometry of this class of anticancer agents. Conjugate 11, in which the TFO is linked via a hexamethylene bridge to the O-4 on the D ring of the anthraquinone moiety, affords the most stable triple helix, through intercalation of the planar chromophore between DNA bases and binding of both the TFO and the amino sugar to the major and the minor groove respectively.

INTRODUCTION

Synthetic oligonucleotides can be used to specifically inhibit the expression of a gene through association with mRNA (the antisense approach) or DNA (the antigene approach). In the former case, the oligonucleotide binds to a Watson–Crick complementary segment of the nucleic acid and interferes, in several ways, with synthesis of the corresponding protein (1,2). Binding of an oligonucleotide (ODN) to double-stranded DNA may occur only at homopurine:homopyrimidine regions and leads to formation of a local triple helix (triplex). The ODN and the duplex are held together by Hoogsteen or reverse Hoogsteen hydrogen bonds between the polypurine strand of the target and the synthetic oligomer, which lies in the major groove. When the triplex-forming oligonucleotide (TFO) has a homopyrimidine sequence, its orientation must be parallel to the purine strand of the target DNA and cytosine protonation is required to form the isomorphous base triplets **C^+·G:C** and **T·A:T** (3). The presence of the triple helical structure can affect replication and transcription of the gene by various mechanisms (3). The association of the third strand with the duplex is much weaker than that which occurs between single strands in the antisense methodology and, in order to form stable triplexes at physiological temperature, it is quite common to link intercalating agents to one or both end(s) of the synthetic oligonucleotide through a spacer unit (3–6). Acridine and psoralen, attached to the 5′-terminus of a TFO via a penta- or hexamethylene linker, were found to intercalate at the triplex–duplex junction (6,7). Besides, if the appended ligand is capable of reacting with or promoting reactions at the binding site, coupling to an appropriate oligonucleotide allows the DNA-damaging activity to the targeted gene to be directed and limited (8–11).

Anthracyclines, widely used and very effective anticancer agents (12), are well-characterized DNA intercalators, showing binding constants in the 10^6–10^7/M range (13). Crystallographic structures have been reported for daunorubicin (Fig. 1) and several other anthracyclines bound to oligodeoxynucleotides (14–16). In all cases, the aglycone moiety was found to intercalate with the long axis nearly perpendicular to the long axis of the adjacent base pairs; ring D protrudes into the major groove, while the hydroxyl group on ring A is anchored by two direct hydrogen bonds to the DNA within the minor groove, which is also the binding site of the sugar.

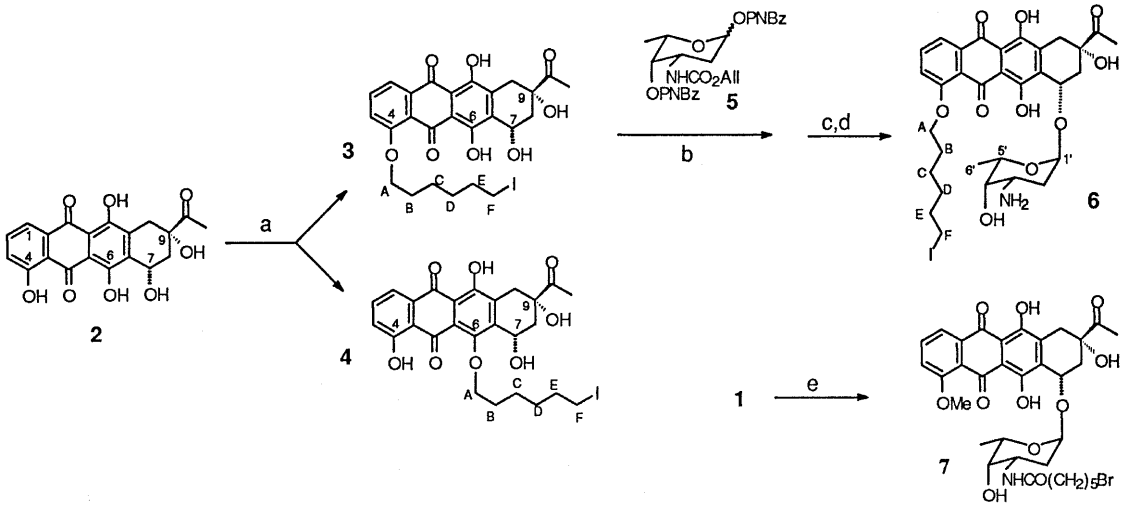
Since a TFO binds to the major groove of DNA, we reasoned that its best attachment site on an anthracycline molecule should be ring D, if the purpose is to maintain, as much as possible, the binding features of the drug which underlie its high affinity for DNA. In addition, this conjugation strategy would allow the delivery of an ‘almost intact’ molecule of an anthracycline antibiotic to a specific gene. To test the validity of the foregoing hypothesis, we prepared four different conjugates by attaching the 5′-end of a homopyrimidine dodecamer, via a hexamethylene bridge, to different sites of daunorubicin and of the corresponding aglycone and compared their affinity for the DNA target with that of the underivatized oligonucleotide by UV and fluorescence spectroscopy.

RESULTS

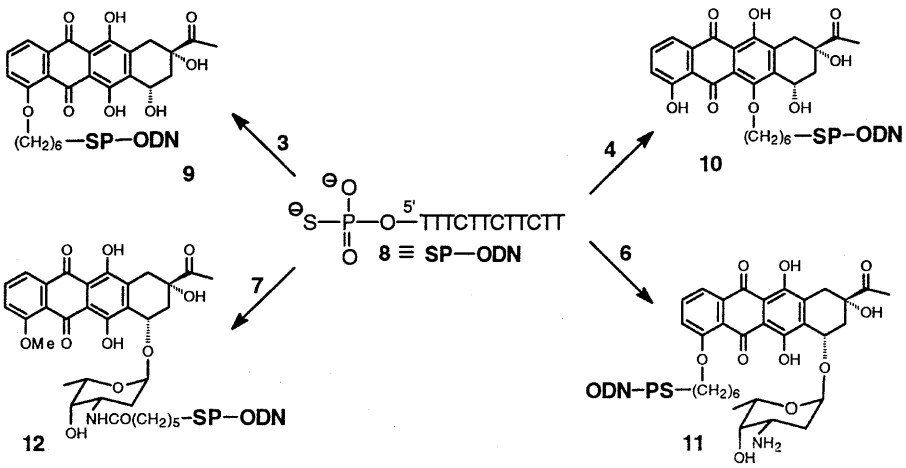
Synthesis

Different strategies have been developed for the design and synthesis of oligonucleotides covalently attached to intercalating molecules (17). In this work the conjugates were obtained upon reaction of an ω-halogenohexamethylene derivative of the anthracycline with the deprotected oligonucleotide carrying a

*To whom correspondence should be addressed. Tel: +39 51 6398306; Fax: +39 51 6398349; Email: garbesi@area.bo.cnr.it



Scheme 1. Synthesis of iodoalkyl derivatives of daunorubicin. Reaction conditions: (a) I-$(CH_2)_6$-I/Ag_2O; (b) TMSOTf/CH_2Cl_2/Et_2O; (c) 0.5 M K_2CO_3/MeOH; (d) $Pd(PPh_3)_4$/2-methylbutyric acid; (e) *p*-nitrophenyl-6-bromohexanoate/*N*-ethyldiisopropylamine. The numbering system used is indicated.



Scheme 2. Synthesis of oligodeoxynucleotide conjugates.

5′-thiophosphate group. The reaction sequence used for the preparation of halogenoalkyl derivatives **3**, **4**, **6** and **7** is summarized in Scheme 1.

Carminomycinone (**2**) (18) was treated with a large excess of 1,6-diiodohexane in refluxing chloroform in the presence of silver oxide (19). The alkylation reaction was sluggish and accompanied by partial aromatisation of ring A. Column chromatography of the crude reaction mixture afforded, in comparable yield, compounds **3** and **4**, identified by mass spectroscopy and ^{1}H- and ^{13}C-NMR. Glycosidation of compound **3** was easily achieved upon reaction with the daunosamine derivative **5** (20) in the presence of trimethylsilyl triflate (21); after a two-step deprotection (20), the purified daunorubicin derivative **6** was obtained in 30% overall yield. Under the same glycosidation conditions, compound **4** did not react with **5**, in agreement with the structure assigned to **4**, which carries the long side chain in a peri position to the reaction centre. ω-Bromohexanoyl derivative **7** was prepared, according to a published procedure (17), by allowing daunorubicin to react with *p*-nitrophenyl-6-bromohexanoate in the presence of a tertiary amine. The conjugates **9–12** were obtained (Scheme 2) by reaction of the sodium salt of 5′-thiophosphate-dodecanucleotide **8** with 10 equivalents of the halogenoalkyl derivatives **3**, **4**, **6** and **7** in DMF/H_2O and in the presence of 15-crown-5 (7); the yields, after HPLC purification, were ~30%.

Binding experiments

UV thermal denaturaturation. The affinities of the conjugates and dodecamer **13** for the DNA target **14** were evaluated by UV melting experiments.

13: 5′-**TTT CTT CTT CTT**-3′
14: 5′-AGG AGC **AAA GAA GAA GAA** CTT T-3′
3′-TCC TCG **TTT CTT CTT CTT** GAA A-5′

Since the triplex-forming sequence **13** contains cytidines, its binding strength is pH dependent (3), hence, the thermodynamic stabilities of the triple helical complexes were monitored at pH 5.5,

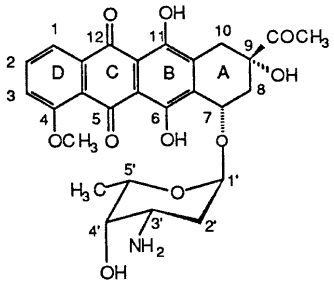


Figure 1. Daunorubicin.

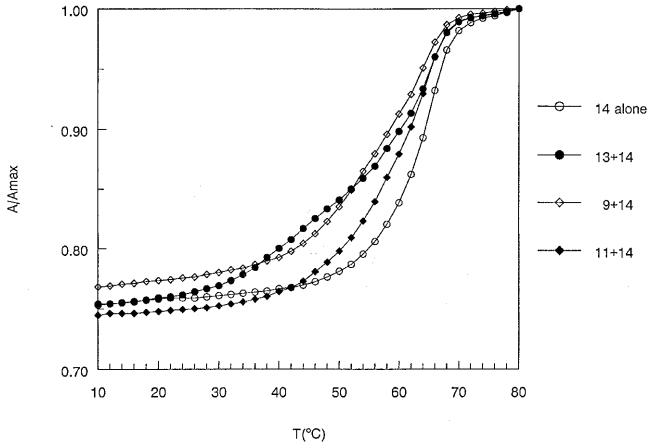
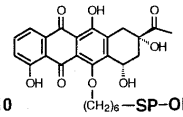
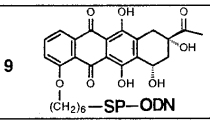
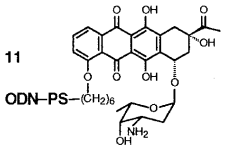
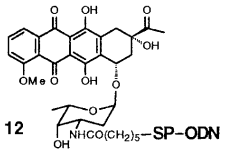


Figure 2. Melting profiles at pH 5.5 of duplex **14** and of the triplexes formed with **13**, **9** and **11** (2.3 μM/strand) in a buffer containing 0.1 M NaOAc, 0.05 M NaCl, 0.01 M $MgCl_2$.

Table 1. Melting temperature values in °C of triple helices at different pH values

compound	T_m at pH 5.5	T_m at pH 6.5 (ΔT_m)	T_m at pH 6.8 (ΔT_m)
TTTCTTCTTCTT (**ODN**) **13**	41	23	**13**
 **10**	>55	43 (+20)	**31** (+18)
 **9**	~51	35 (+12)	**27** (+14)
 **11**	>55	~45 (+22)	**36** (+23)
 **12**	~45	25 (+2)	**16** (+3)

Values were obtained from the position of the maximum of the first derivative of the corresponding melting profiles. ΔT_m is the difference between the melting temperature of the conjugate and that of **13** at the same pH. In all cases the melting temperature of the duplex was found to be 65°C.

6.5 and 6.8. The denaturation process was followed by recording the change in UV absorbance with temperature at 260 nm. As shown in Figure 2, at the most acidic pH the melting curves failed to show a clear biphasic profile. Formation of the triple helical complexes was therefore probed by gel electrophoresis at 15°C (not shown). On the other hand, well-defined biphasic profiles were obtained at pH 6.8. The corresponding triple to double helix transitions are shown in Figure 3.

The T_m values reported in Table 1 show that the thermal stability of the triple helix is enhanced when a daunorubicin derivative is attached at the 5′-end of oligonucleotide **13**. However, the contribution of the appended ligand depends, quite strongly, on both its chemical structure and the attachment site of the bridge unit. This effect is particularly important at pH 6.8, where the contribution of the oligonucleotide moiety to the binding strength is greatly reduced.

Fluorescence quenching. It is known that the fluorescence of anthracyclines is quenched by intercalation within the DNA base pairs (22,23). The fluorescence emission spectra (520–700 nm) at 25°C of the triple helical complexes were first recorded at pH 5.5 and compared with those obtained after increasing the pH of the solutions to 8.2, a condition which is not compatible with the existence of the type of triplex in hand. In all cases, the intensity of the spectrum at pH 8.2 was much higher than that obtained at pH 5.5, while that of the conjugates alone was practically the same at both pH values. In fact, the intensity of the spectrum recorded after raising the pH of the triplex solution to 8.2 was comparable with that of the conjugate under the same experimental conditions in the absence of the DNA duplex. The results obtained with conjugate **11** are shown in Figure 4, in which the spectrum of the same solution recorded at pH 5.5 (triple helix), at pH 8.2 (double helix + free conjugate) and 1 min after again lowering the pH to 5.5 are presented. The spectrum recorded after standing at 25°C for ~30 min was practically identical to that of the starting triple helix.

Fluorescence spectroscopy was also used to prove that the binding site recognition properties of the conjugates, which are bifunctional DNA ligands, are exclusively dictated by the oligonucleotide component. Daunorubicin binds very strongly to double helical oligonucleotides containing the 5′-CGT sequence (24). In fact, when a micromolar solution of the drug was titrated with increasing amounts of $d(CGTACGTACG)_2$ the initial fluorescence was almost completely quenched after addition of 1 M equivalent of the duplex (Fig. 5a). When this latter was added to a solution of conjugate **11**, the starting spectrum remained unchanged (Fig. 5b).

DISCUSSION

As expected, the attachment of an anthracycline derivative to the 5′-end of dodecamer **13** led to an increase in the affinity of the ODN–anthracycline conjugate for the homopurine–homopyrimidine duplex in comparison with the unmodified ODN. For each derivative the half-dissociation temperatures at

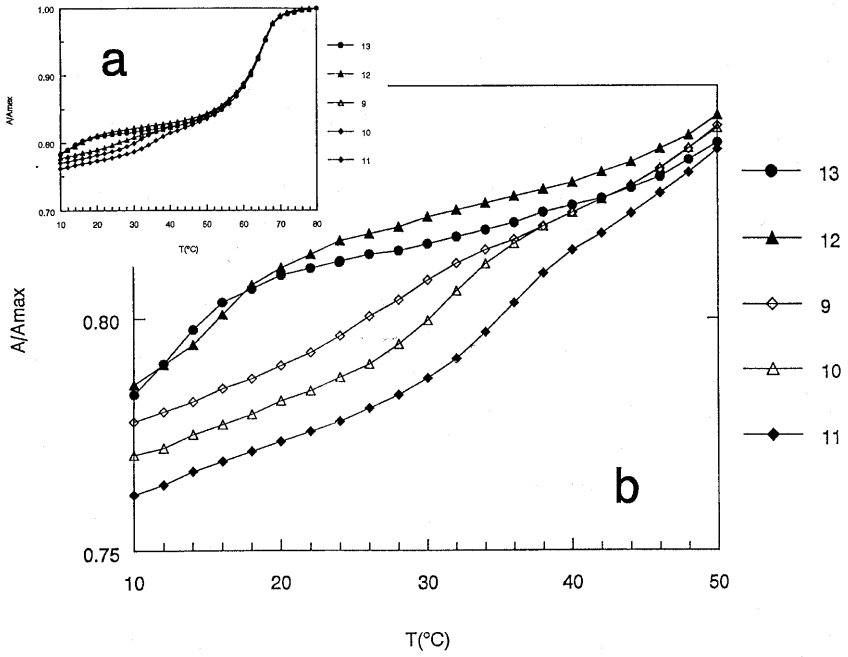


Figure 3. (**a**) Melting profiles at pH 6.8 of the triplex formed by duplex **14** with underivatized ODN **13** and conjugates **9–12**. Each strand is 2.3 µM in a buffer containing 0.1 M NaOAc, 0.05 M NaCl, 0.01 M $MgCl_2$. (**b**) Magnification of the triplex to duplex transition.

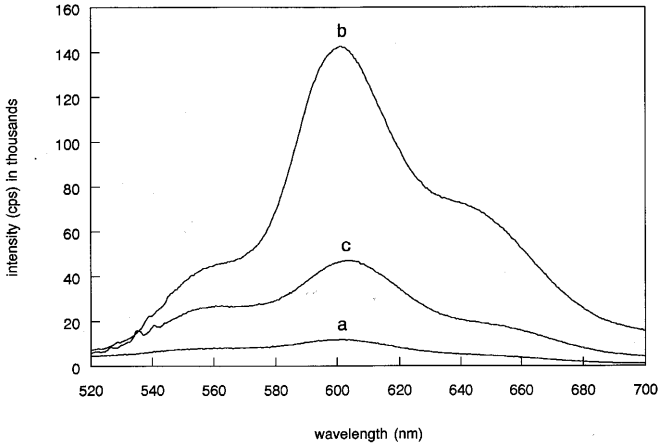


Figure 4. Fluorescence spectra at 25°C of the solution (0.1 M NaOAc, 0.05 M NaCl, 0.01 M $MgCl_2$) containing duplex **14** and conjugate **11** (2.3 µM/strand) at (**a**) pH 5.5, (**b**) pH 8.2 and (**c**) again at pH 5.5 1 min after acidification.

pH 6.5 and 6.8 show that the gain in thermal stability is independent of pH (see Table 1) and the fluorescence quenching experiments at pH 5.5 demonstrate that, with all conjugates, the daunorubicin chromophore is intercalated within the bases of the triple helical complexes. However, as expected, the contribution of the appended ligand depended upon its chemical nature and the site of attachment of the linker unit. Anthracyclines are composed of two main structural constituents: the aglycone, which contains the intercalating anthraquinone moiety, and the amino sugar. The T_m values of the triple helices formed by conjugates **9** and **10** show that the aglycone tethered at the 5′-end of the oligonucleotide strongly enhanced the affinity for the target duplex and that the gain in stability depended upon the site of attachment of the linker. The observed increases in the half-dissociation temperature are comparable with those reported for acridine-linked oligonucleotide, whose length and base composition are similar to those of sequence **13** (5). However, a further increase in stability (ΔT_m = 9°C) was obtained if the amino sugar moiety was attached to the intercalating chromophore. Comparison of the melting temperatures of the complexes formed by unmodified **13** and conjugates **9** and **11** indicates that the gain in stability following attachment of the oligonucleotide to position 4 of daunorubicin was due, by about one third, to the daunosamine portion of the appended molecule. Thus, **11** binds to DNA by intercalation and simultaneous recognition of the major and the minor groove. When the oligonucleotide was connected to the amino group of the sugar (derivative **12**), the increase in the melting temperature was almost negligible. Since the amino group of the drug is protonated even at neutral pH, its derivatization was expected (25) to reduce the affinity of conjugate **12** in comparison with **11**, but the experimental results show that the contribution of the aglycone moiety was almost completely lost. It is reasonable to ascribe this finding to the overall structure of conjugate **12**, which, because of mandatory binding of the oligonucleotide in the major groove, forces the daunorubicin moiety to reverse its natural intercalation geometry (see Introduction). As a matter of fact, a recent study on the binding of free anthracycline derivatives to DNA showed that both the substituent(s) on ring A and the sugar moiety make a large contribution to the affinity upon binding to the minor groove and that alteration of the stereochemistry of the daunosamine residue has a high energetic cost (13). Moreover, while it is reasonable to assume that intercalation takes place at the triplex–duplex junction with compounds **9–11**, by analogy with acridine and psoralen conjugates featuring penta- or hexamethylene linker (6,7), intercalation of the anthraquinone chromophore of conjugate **12** may possibly occur at different sites, because of the larger distance between the binding regions

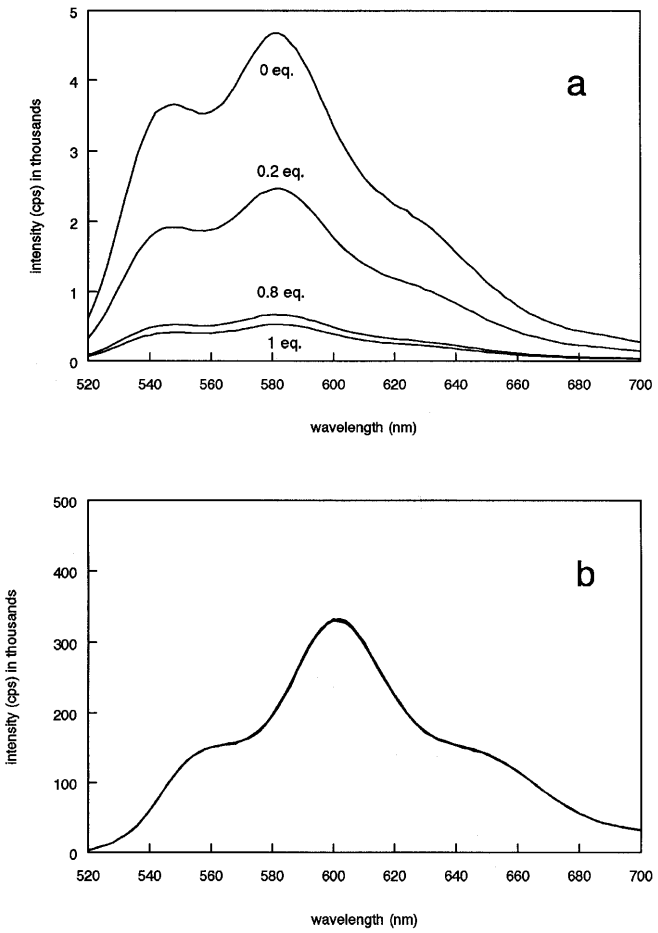


Figure 5. (**a**) Quenching of fluorescence of daunorubicin **1** (2.3 µM) with the indicated equivalents of d(CGTACGTACG)$_2$ at pH 7.0 and 25°C in the presence of 0.1 M NaOAc, 0.05 M NaCl, 0.01 M $MgCl_2$. (**b**) Superimposition of spectra of conjugate **11** (2.3 µM) with 0, 0.2, 0.4 and 1.0 equivalents of d(CGTACGTACG)$_2$ in the same buffer as above at 25°C.

(5). In summary, the experimental results show that the covalent attachment of a TFO, via an appropriate linker, to position 4 or 6 of the aglycone moiety of daunorubicin strongly enhances the affinity for the target duplex. Moreover, it is demonstrated that the daunomycin derivative itself adds a further stabilizing interaction when the bridging unit is attached to ring D. In fact, conjugation of an anthracycline to a TFO can be viewed as a means to deliver these anticancer drugs to a specific target within the genome, since, as proved by the fluorescence quenching experiments, intercalation occurs *only* where the DNA contains the binding site for the oligonucleotide. Anthracyclines are amongst the most widely used and effective anticancer drugs. Despite over 25 years of inquiry, the mode of action of these cytotoxic molecules is still a matter of debate, but a fair agreement exists as to the importance of intercalation into double helical DNA. Following intercalation, three main mechanisms of action are currently envisaged. The first is based on the ability of the drug to interfere with the activity of topoisomerase II, a nuclear enzyme required for DNA replication and transcription, ultimately leading to formation of a 'cleavable complex' and subsequent DNA strand breakage (26,27). The second mechanism, which has been proposed for the very potent 3′-morpholinyl derivatives, entails the alkylation of DNA by reactive species deriving from the morpholinyl groups (28). The third is based on the hypothesis that the anthraquinone system promotes formation of hydroxyl radicals that, in turn, cause DNA strand scission (29). While the topoisomerase-mediated activity of the drug will most likely be abolished by the presence of a triple helix next to the intercalation site (30), the other two mechanisms should not be substantially altered when, as in conjugate **11**, the drug is attached to the oligonucleotide in a way that leaves its chemical features and its intercalation geometry almost unchanged.

MATERIALS AND METHODS

General methods

All chemicals were of analytical grade and were used as received, unless otherwise stated. Carminomycinone (**2**) and 1,4-di-*O*-*p*-nitrobenzoyl-*N*-allyloxycarbonyl-L-daunosamine (**5**) were a gift from F.Animati, A.Cipollone M.Berettoni and M.Franciotti (Menarini Ricerche). *N*-(6-Bromohexanoyl)-daunorubicin (**7**) was prepared according to the literature (17). The compound gave a satisfactory ^{1}H-NMR spectrum. Solvents used for reactions were purified by distillation and stored over molecular sieves. The oligodeoxynucleotides were prepared on a Pharmacia Gene Assembler II-Plus using the manufacturer's protocols with commercially available amidites, but 0.75 M ethylthiotetrazole in CH_3CN, instead of 0.5 M 1-H-tetrazole, was used during the whole synthesis (32). TLC analysis was conducted on Merck 5719 silica gel (230–400 mesh) plates and flash chromatography was performed on Merck 9385 silica gel (230–400 mesh). NMR spectra were recorded with either a Varian VXR 200 or a Varian Gemini 300 spectrometer, using TMS and 85% H_3PO_4 as external standards, as references for ^{1}H and ^{31}P respectively. Mass spectra were recorded on a VG Quattro spectrometer. Electrophoreses were run using 20% polyacrylamide gels in a thermostatic slab gel unit at 10 V/cm. Gel staining was obtained by soaking the gel with 0.01% stain-all dye in 1/1 (v/v) water/formamide.

HPLC analysis and purifications

HPLC analysis and purifications were performed on a Waters 600E Millipore system control equipped with a Waters 484 tunable absorbance detector. We have used the methods listed below.

Method 1 (ionic exchange). HPLC was run over a TOSOHAAS TSK gel DEAE-5PW column (7.5 mm i.d. × 15 cm) using the eluents A (0.02 M NaOAc, 0.02 M $NaClO_4$ in H_2O/CH_3CN, 9/1) and B (0.02 M NaOAc, 0.3 M $NaClO_4$ in H_2O/CH_3CN, 9/1). A linear gradient from 100% A to 50% A/50% B over 50 min was applied at a flow rate of 1 ml/min.

*Method 2 (reverse phase analysis and purification of conjugates **9–12**).* HPLC was run over a TOSOHAAS TSK gel OD-2PW column (4.6 mm i.d. × 15 cm), eluting with A (0.1 M triethylammonium acetate in H_2O, pH 7.2) and B (CH_3CN) at a flow rate of 1 ml/min; λ = 260 nm. The gradient was: t = 0, A/B, 95/5; t = 20, A/B, 67/33; t = 22, A/B, 20/80; t = 38, A/B, 20/80; t = 42, A/B, 95/5; t = 50, A/B, 95/5.

Method 3 (preparative reverse phase). A Lichrospher RP-18 7 µm (25 mm i.d. × 25 cm) column was used. Elution was performed

isocratically with CH_3CN/H_2O/triflouroacetic acid, 90/10/0.1 at a flow rate of 10 ml/min; λ = 234 nm.

UV melting experiments

UV measurements and melting profiles were recorded on a Perkin Elmer 554 spectrophotometer equipped with a MGW Lauda RC5 thermostat and a MGW Lauda R40/2 digital thermometer; heating was controlled by an electronic device generating a linear temperature gradient of 0.5°C/min. Samples were prepared by dissolving equimolar amounts of each strand in 0.1 M NaOAc, 0.05 M NaCl, 0.01 M $MgCl_2$ at pH 5.5, 6.5 and 6.8 (increasing the pH with NaOH) to a final concentration of 2.3 μM/strand. The UV cuvettes were heated to 80°C for 20–30 min then slowly cooled (over at least 2–3 h) to 4°C to favour annealing. Condensation of moisture on the cell walls was prevented by flushing with nitrogen.

Fluorescence experiments

Fluorescence quenching experiments were monitored on a SPEX 3000 spectrofluorimeter at room temperature with 1.25 mm slits and 455 nm and 495 nm filters, λ_{exc} = 470 nm; the emission was recorded in the interval 520–700 nm. Interference of water was electronically subtracted from the recorded spectra. Where possible, the same samples used for the UV measurements were used for registering the fluorescence spectra.

4-*O*-(6-Bromohexyl)-carminomycinone (3) and 6-*O*-(6-bromohexyl)-carminomycinone (4)

Carminomycinone (**2**) (18) (5.0 g, 13 mmol) was partially dissolved in 650 ml $CHCl_3$ then 35 g (104 mmol) 1,6-diiodohexane were added together with 6.9 g (29.9 mmol) Ag_2O. The reaction mixture was stirred under nitrogen at reflux temperature. Every day 1.6 g (6.9 mmol) fresh Ag_2O were added until TLC analysis (CH_2Cl_2/acetone, 96/4 v/v) showed complete disappearance of carminomycinone (usually 3–5 days). Two major compounds were formed: a yellow one (**4**) (R_f = 0.50), corresponding to the product formed upon alkylation of O-6, and a red one (**3**) (R_f = 0.33), corresponding to that formed upon alkylation of O-4. The reaction mixture was filtered in order to remove the silver salts, then concentrated to a small volume. The reaction products, recovered as a precipitate after addition of hexane, were purified by silica gel column chromatography in CH_2Cl_2 with an acetone gradient from 0 to 2%. Compounds **3** and **4** were isolated in 12% (925 mg) and 15% (1.15 g) yield respectively.

3. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.55 (m, 2H-C, 2H-D), 1.95 (m, 2H-B, 2H-E), 2.15, 2.35 (2d, 2H-8), 2.40 (s, Me-14), 9.92, 3.18 (2d, 2H-10), 3.21 (d, 2H-F), 3.69 (d, OH-7), 4.19 (t 2H-A), 4.51 (s, OH-9), 5.23 (bs, H-7), 7.34 (d, H-3), 7.71 (t, H-2), 7.99 (d, H-1), 13.24 (s, OH-6). 14.09 (s, OH-11). ^{13}C-NMR (300 MHz, DMSO-d_6): δ 8.9 (F), 24.2, (14), 28.3, 29.4, 32.0, 32.7 (C, D, B, E, 10), 35.9 (8), 60.5 (7), 68.9 (A), 76.2 (9), 110.3 (5a, 11a), 118.6 (1), 120.3 (3), 133.2, 134.2 (10a, 12a), 135.8 (2), 154.4, 155.5 (6, 11), 159.9 (4), 186.0, 186.1 (5, 12), 211.3 (13). FAB-MS: M^+ found 594 (calculated 594.4)

4. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.58 (m, 2H-C, 2H-D), 1.91, 1.89 (m, 2H-B, m,2H-E), 2.16, 2.34 (2d, 2H-8), 2.41 (s, Me-14), 2.96, 3.17 (2d, 2H-10), 3.22 (t, 2H-F), 3.94 (m, 1H-A and OH-7), 4.24 (q, 1H-A), 4.56 (s, OH-9), 5.23 (bs, H-7), 7.32 (d, H-3), 7.64 (t, H-2), 7.96 (d, H-1), 13.02 (s, OH-4), 13.74 (s, OH-11). ^{13}C-NMR (300 MHz, $CDCl_3$ assignation by HETCOR): δ 7.0 (F), 24.8 (14), 29.7 (C, D), 29.9 (B, E), 33.3 (10), 35.7 (8), 62.2 (7), 75.4 (A), 77.0 (9), 114.0 (5a, 11a), 117.0 (4a), 118.8 (1), 125.2 (3), 132.6 (10a, 12a), 136.2 (2), 142.0 (6a), 152 (6), 158.8 (11), 162.7 (4), 187.0 (5, 12), 208.0 (13). FAB-MS: found 594 (calculated 594.4).

4-Demethyl-4-*O*-(6-iodohexyl)-daunorubicine hydrochloride (6)

A sample of 594 mg (1 mmol) **5** and 624 mg (1.18 mmol) **3** were mixed and kept for 1 day under vacuum at 40°C. To the mixture, co-evaporated three times under reduced pressure with dry toluene, 6 g 4 Å activated molecular sieves were added under nitrogen, followed by 200 ml CH_2Cl_2/Et_2O, 3/1. The mixture was cooled to –5°C and 0.436 ml (2.37 mmol) trimethylsilyltriflate were added. After 5 min the reaction was quenched with 50 ml 2% $NaHCO_3$. The molecular sieves were removed and the organic phase was diluted with 100 ml CH_2Cl_2, washed with water, dried over Na_2SO_4 and then evaporated *in vacuo*. After purification by HPLC (method 3), 570 mg (52%) protected glycoside were isolated. To 570 mg (0.52 mmol) protected glycoside dissolved in 200 ml MeOH/CH_2Cl_2 (77/13) and cooled to –10°C under nitrogen, 4.2 ml (2 mmol) 0.5 M K_2CO_3 were added and the reaction was followed on TLC ($CHCl_3$/^{i}PrOH, 95/5). After 1 h the reaction mixture was neutralized with 0.1 M HCl (the colour of the solution changes from violet to orange), the organic phase was washed with water, dried with Na_2SO_4 and evaporated (HPLC yield 92%).The residue was purified by HPLC (method 3) to give 309 mg partially deprotected glycosides (yield 58%). PPh_3 (8.1 mg, 0.031 mmol), $Pd(PPh_3)_4$ (12.8 mg, 0.011 mmol) and 2-methylbutyric acid (96 μl 0.87 mmol) were added to a solution of 309 mg (0.31 mmol) partially deprotected glycoside in anhydrous CH_2Cl_2 (67 ml). The reaction was kept in the dark under stirring for 3 h, monitored by TLC ($CHCl_3$/MeOH/HCOOH/H_2O, 79/9/2/1). The reaction mixture was concentrated to half volume, then extracted with portions of 10 ml 10^{-4} M HCl until a colourless extract was obtained. The combined aqueous extracts were washed with AcOEt, adjusted to pH 4.0 with dilute $NaHCO_3$ and lyophilized to give 185 mg (80%) **6**. ^{1}H-NMR (300 MHz, $CDCl_3$ after exchange with D_2O): δ 1.33 (d, Me-6′), 1.5–1.7 (m, 2H-C, 2H-D), 1.8–2.0 (m, 2H-B, 2H-E), 2.07, 2.35 (2d H-8), 2.39 (s, Me-14), 2.94, 3.19 (2d, H-10), 3.10 (m, H-3′), 3.22 (t, 2H-F), 3.46 (s H-4′), 4.09 (q, H-5′), 4.17 (t, 2H-A), 5.28 (bs, H-1′), 5.49 (d, H-7), 7.32 (d, H-3), 7.71 (t H-2), 7.97 (d, H-1). FAB-MS: found 724 (calculated 723.6).

Purification of unmodified oligonucleotides

The crude compounds obtained from the automatic synthesis were deblocked with 30% aqueous ammonia in a sealed vial at 50°C for 16 h. The mother solutions were lyophilized and the oligonucleotides were purified by ion exchange on a Pharmacia preparative column of DEAE–Sephacell (2× 15 cm) eluting with a linear gradient of 0.05–1.2 M triethylammonium hydrogen carbonate (~400 ml in total). After HPLC analysis, the appropriate fractions were collected and lyophilized twice, then converted to the sodium salts by passing them through a column of Dowex 50W-8 in the sodium form.

5′-PS-d(TTTCTTCTTCTT) (8)

After the usual synthetic procedure, 4 equivalents of bis-2-cyanoethyl(*N*,*N*-diisopropyl)phosphoramidite (33) (0.1 M in CH_3CN) were recycled for 7 min in the reactor in the presence of ethylthiotetrazole (32) (11 equivalents), then the exhaust mixture was washed away with acetonitrile and the procedure was repeated twice more (17). Sulphurization was performed with the Beaucage reagent, following the protocol suggested by Pharmacia. The modified oligonucleotide was deblocked with 30% aqueous ammonia in a sealed vial at 50°C for 24 h, purified as the unmodified oligonucleotides, converted to the sodium form then passed through a column of Chelex to eliminate traces of divalent metals (17) and lyophilized. The product was 2,6-dibromoparabenzoquinone-*N*-chloroimine test positive (17). ^{31}P-NMR: δ 45.5 p.p.m. (PS linkage) (33), –0.5 p.p.m. (other PO linkages). HPLC analysis: method 1 (ion exchange): 12mer 5′-PS (**8**), R_t = 36.3; (other products: 12mer 5′-OH (**13**), R_t = 34.3 min; 12mer 5′-PO, R_t = 35.0; dimer PS-SP, R_t = 44.7).

Electronspray MS of the sodium salt: [M-3Na]/3 = 1286, giving a mol. wt of 3927 ± 2 (calculated 3925.16). [In comparison, analysis of the 5′-OH 12mer (**13**) and 5′-PO 12mer gave mol. wts of 3789 ± 2 (calculated 3785.16) and 3912 ± 3 (calculated 3909.10).]

Conjugates 9–12

The sodium salt of the 5′-PS-oligonucleotide **8** (10 OD, ~0.1 µmol) was dissolved in 125 µl *N*,*N*-dimethylformamide, 50 µl water, 13 µl 15-crown-5 and 1 mg anthracycline haloderivative **3**, **4**, **6** or **7**. The reaction mixture was kept in a sealed vial for 16 h in a thermostat at 45°C and the reaction was followed by reverse phase HPLC (method 2). The crude mixture was purified from the excess anthracycline using a short cartridge of RP-18 (3× 1 cm), then concentrated and purified by reverse phase HPLC (method 2). The following peaks were assigned in the reaction mixture: oligo 5′-PS **8**, R_t = 11.6 min; oligo 5′-PO (formed during the conjugation), R_t = 12.0; dimer PS-SP (partially formed during the reaction), R_t = 12.5; **9**, R_t =16.5; **10**, R_t =17.3; **11**, R_t = 17.1; **12**, R_t = 16.2.

UV characterization

All conjugates showed an UV profile characterized by two broad absorption maxima: one at 260 nm due to the oligonucleotidic moiety and to the anthracycline system, the second in the visible portion of the spectrum due to the anthracycline alone (see for example Fig. 6). We made the assumption that the spectra of the conjugates are the sum of the spectra of the separate components, the oligonucleotide and the anthracycline derivative. We also assumed that in compounds **9–12** the tethered daunorubicin derivatives contribute 20 500/M/cm to the molar absorption of the conjugates at 260 nm, as deduced from the UV spectrum of doxorubicin in water, so that the resulting molar absorption of the conjugates was 115 300/M/cm (94 800/M/cm being the calculated absorption of the oligo alone) (33). This value was used to titrate the mother solution of the conjugates for the melting experiments. The position and value of the absorbance at visible wavelengths varied for the different compounds. The following absorption ratios were recorded: conjugate **9**, A_{260}/A_{520} = 11; conjugate **10**, A_{260}/A_{452} = 15; conjugate **11**, A_{260}/A_{490} = 12; conjugate **12**, A_{260}/A_{490} = 9.

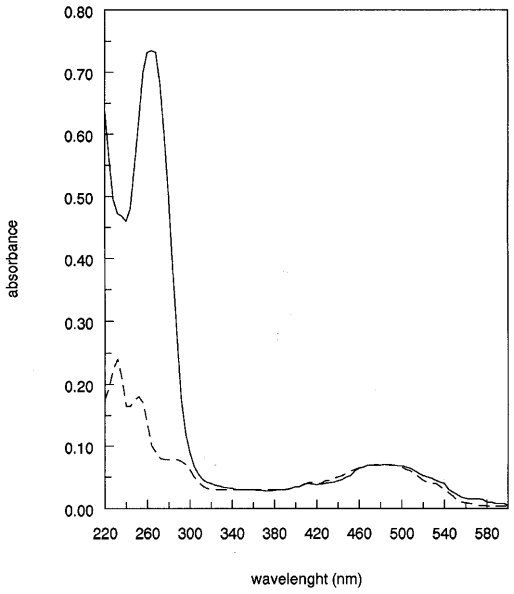


Figure 6. UV and visible spectrum of doxorubicin (dashed line) and conjugate **11** (full line) in water at 25°C.

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