First Route to Phosphoramidites of N^2-Hydrazinoaryl- and N^2-Azoaryl-dG Adducts and Their Site-Selective Incorporation into DNA Oligonucleotides

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Abstract: N^2-Hydrazinoaryl and N^2-azoaryl-dG adducts of borderline carcinogens and the bladder and breast carcinogen 4-aminobiphenyl were prepared using Pd-catalyzed cross-coupling chemistry. After conversion into the phosphoramidites site-specifically damaged oligonucleotides were prepared by automated DNA synthesis. DNA Hybrids were studied with respect to their thermal stability (UV melting-temperature analysis) and circular dichroism.

Key words: DNA damage, chemical carcinogenesis, aromatic amines, N^2-adducts, cross-coupling

DNA contains the blueprint for the proper development, functioning, and reproduction of every organism. Alterations affecting the structure and integrity of DNA molecules can arise spontaneously through intrinsic instability of chemical bonds in DNA or can be induced by irradiation, oxidative stress, or chemical carcinogens.[1] Exposure to carcinogens can occur from environmental or working conditions, diet, smoking, and endogenous processes. Poly- and monocyclic aromatic amines belong to the class of chemical carcinogens that may form covalently bonded adducts with DNA. Covalent alteration of DNA (by electrophiles) may be the reason for the induction of chemical carcinogenesis.[2] If these covalently bonded modifications are not repaired, they might compromise the fidelity of DNA replication, leading to mutations and possibly cancer.[3,4] Prior to adduct formation, the aromatic amines underlie a metabolic activation that works parallel to the detoxification process. Ring oxidation and N-acetylation normally led to detoxification. However, N-oxidation catalyzed by monooxigenase cytochrome P450 results in the formation of *N*-arylhydroxylamines. If these are esterified, e.g. by acetyl-CoA to yield *N*-acetoxyarylamines, highly electrophilic reagents are formed that react with bionucleophiles, e.g. the DNA base guanine.[5] It is assumed that *N*-acetoxyarylamines form nitrenium ions which react in an electrophilic amination reaction with nucleophilic centers. Thus, the latter metabolite act as a so-called ultimate carcinogen.[6] As major product with DNA, modification of the C8-position of 2′-deoxyguanosine as shown in adduct type **1** (dG) was identified in vivo.[7] In lower extent the corresponding 2′-deoxyadenosine (dA) adduct was also observed.[8] Moreover, adducts at the nucleophilic N^2-position as shown in the hydrazino compound **2** and/or the azoaryl-compound **3** of dG were identified as well in in vivo studies and thus may have considerable biological relevance. These adducts have not been investigated in detail due to missing synthetic access (Figure 1).[9] In addition, also the N^2-arylamine dG adducts in which the bond is formed between the N^2 atom and the *ortho* C atom of the arylamine were found.

In contrast to the biological impact of polycyclic *N*-arylamines, little is known of their monocyclic counterparts, which are classified as borderline carcinogens. Borderline carcinogens are less mutagenic/carcinogenic than their strongly carcinogenic counterparts. Often they express their carcinogenicity only under specific circumstances, e.g. high and long-lasting exposition to these compounds. However, the reasons for this difference remain still unclear. Thus, we focused our interest to DNA lesions caused by monocyclic aromatic amines like aniline, toluidine, or 4-anisidine.[10,11]

So far, most studies have been done with C8-arylamine adducts and the properties of damaged DNA strands by this type of adduct. We and others have reported on syn-

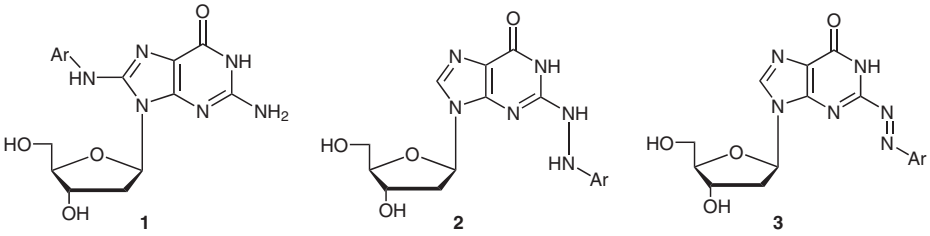

Figure 1 In vivo formed nucleobase adducts of arylamines with 2′-deoxyguanosine

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thetic approaches to such modified adducts using not only borderline carcinogens, but also poly- and heterocyclic arylamines, the site-specific incorporation into oligonucleotides as well as the effect on thermal stability, conformation, and replication.[12–16]

In addition to the C8-modification we were interested in N^2-arylamine adducts, which should interfere with the interstrand hydrogen-bond pattern within the DNA double helix.

In 1999 de Riccardis et al. published a first synthetic route to N^2-arylamine adducts of 2′-deoxyguanosine, in which the aromatic ring was bound to the exocyclic amino function via a C–N bond.[17] Using heterocyclic amines Rizzo published the first incorporation of such adducts into oligonucleotides.[18] Again the arylamine was linked via an arylamine C atom to the exocyclic amino group of dG.

In 2004 Boche et al. reported on the synthesis of N^2-(4-dG-aminobiphenyl)-3′-phosphates using a substitution of triflated 2′-dG derivative with 4-hydrazinobiphenyl. However, the product was isolated only in moderate yields.[19] Application of that method to the preparation of the adduct phosphoramidite failed to obtain sufficient amounts necessary for the coupling reaction.

Here, we disclose the first synthesis of N^2-hydrazinoaryl- and N^2-azoaryl-dG-3′-*O*-phosphoramidites and their incorporation into oligonucleotides by automated DNA synthesis. In these adducts, the amino function of the arylamine is directly bound to the exocyclic amino group via an N–N bond of dG as shown in compounds **2** and **3** (Figure 1).

In contrast to Boche's approach, our synthesis is based on a palladium-catalyzed cross-coupling approach. Therefore, it became apparent that the two hydroxyl groups as well as the O^6-position of dG **4** have to be protected.

Thus, first O-silylation using TBDMSCl was performed to block the hydroxy groups of the 2′-deoxyribose and Pfleiderer's β-cyanophenylethyl (CPE) group was introduced as O^6-protection (Scheme 1).[20] Finally, the exocyclic amino function was converted into bromide **5** using $SbBr_3$ and *tert*-butylnitrite (3 steps, 47% overall yield). 2-Bromopurine nucleoside **5** is the key intermediate for the cross-coupling reaction leading to the N^2-adducts.

The subsequent cross-coupling reaction of bromide **5** and arylhydrazines **6a–d**, which are commercially available or can be easily synthesized,[21] was carried out using previously published conditions.[13] However, here the addition of triethylammonium chloride[22] to the reaction mixture increased the yield of adduct **7a–d** from 14–32% up to 52–80% (Scheme 1).[23,24]

To convert N^2-arylamine adducts **7a**,**b** into the corresponding phosphoramidites, first the silyl ethers were cleaved using triethylammonium trihydrofluoride. During the separation of product **8a**,**b** using silica gel chromatography the oxidation products **9a**,**b** were formed as well (Scheme 1). Due to the fact that this type of adduct also plays a role in the possible induction of the chemical carcinogenesis by aromatic amines both compounds **8a** and **9a** were successfully separated by RP silica gel column chromatography and isolated in 31% and 48% yield (hydrazinoaryl adducts **8a**,**b**) and in 25% and 27% yield (azoaryl compounds **9a**,**b**).

Compounds **8a**,**b** and **9a**,**b** were 5′-*O*-dimethoxytritylated and further converted into 5′-*O*-DMTr-3′-*O*-phosphoramidites **10a**,**b** and **11a**,**b** (28–56% yield for both steps).[25]

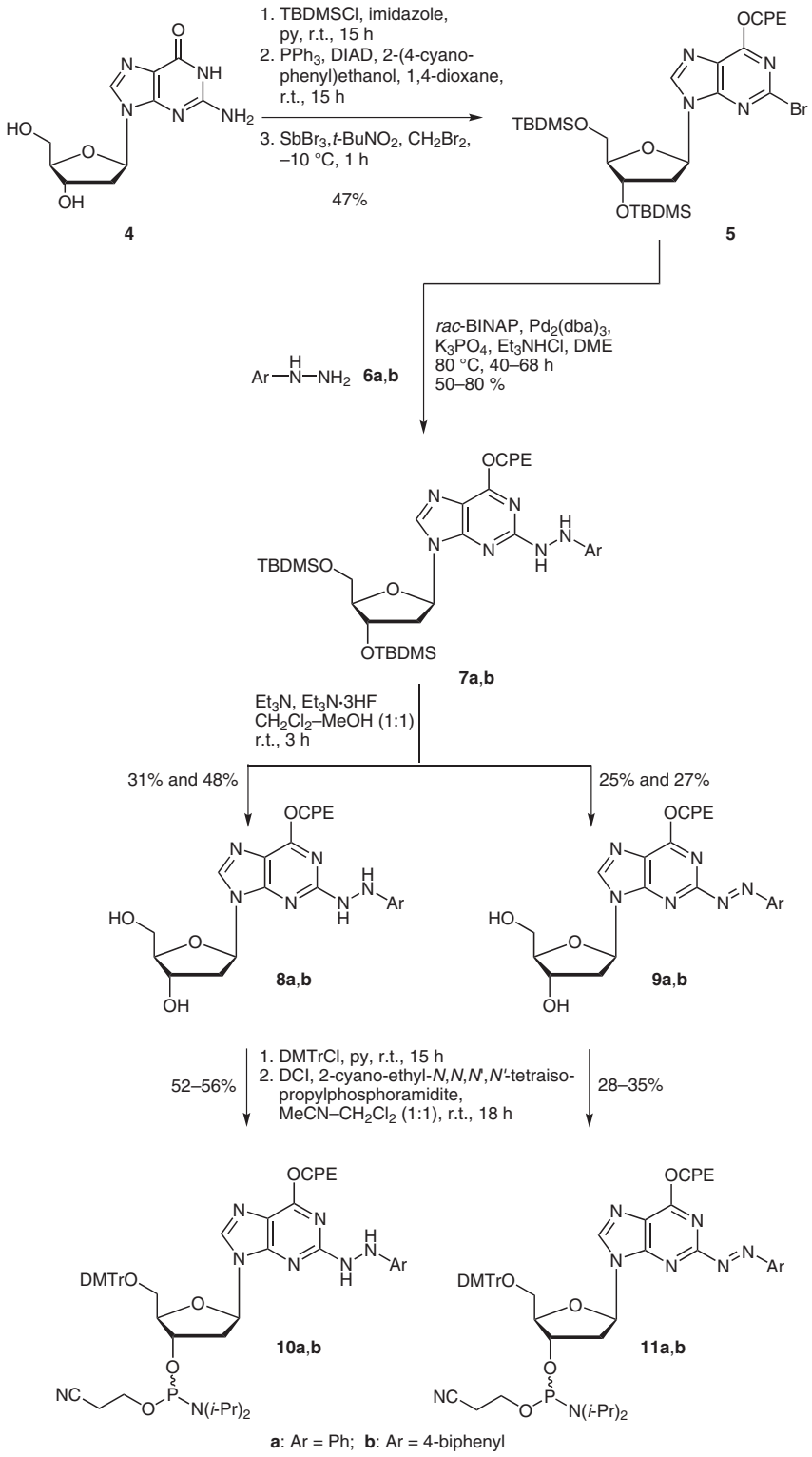
However, if only the hydrazinoaryl adduct is needed, the purification of compounds **8a**,**b** and **10a**,**b** should be done using an aluminium oxide (neutral) column chromatography to avoid the oxidation to the corresponding azoaryl derivative.

Phosphoramidites **10a**,**b** and **11a**,**b** were used in automated oligonucleotides synthesis using a modified coupling protocol. For the site-specific incorporation of these adducts into the 30mer sequence 5′-AAA T(**G***)A ACC TAT CCT CCT TCA GGA CCA ACG-3′ and a self-complementary 12mer 5′-GTA(**G***)AATTCTAC-3′ the phosphoramidites **10** and **11** were dissolved in acetonitrile. The coupling period was tripled compared to the regular phosphoramidite building blocks. The coupling efficiency was about 80% (based on the commonly used trityl assay).

After the synthesis the deprotection of the O^6-CPE group was achieved by treatment with DBU in pyridine (24 h at r.t.).[20] Due to the fact that DBU does not cleave the oligonucleotide from the CPG solid support, the CPG-bond oligonucleotide could be isolated by simple filtration and the cleavage of the base and phosphate protecting groups as well as the CPG support was done using ammonium hydroxide solution and mercaptoethanol.[14] Purification of the oligonucleotides was achieved by reversed-phase HPLC. The oligonucleotides showed high purity as indicated by HPLC and they were characterized by ESI-MS.

All oligonucleotides were hybridized with the complementary strand and the effect of the incorporated N^2-arylamine adducts on the thermal stability of the DNA duplex was measured by UV melting-curve analysis (T_m). In general, the N^2-lesions destabilized the DNA duplex. Disturbance of the helix is most probably due to lower hydrogen-bond stabilization. As expected, the effect on the thermal stability was found to be bigger for the 30mer oligonucleotides that are damaged by the large, strong carcinogen 4-aminobiphenyl (3 °C decrease compared to the unmodified oligonucleotide). The 30mer oligonucleotides modified with the borderline carcinogen aniline showed a moderate destabilization (1.5–2 °C). Interestingly, the effect is not depending on the type of the lesion since there is no significant difference in the thermal stability for the hydrazinoaryl- and azoaryl-damaged oligonucleotide.

The effect of the N^2-damage is more significant in the self-complementary strand. For all N^2-modified oligonucleotides no formation of a DNA duplex could be observed at a temperature of 5 °C and, thus, no T_m value could be measured.



Scheme 1 Synthesis of the phosphoramidites of N^2-dG adducts **10a**,**b** and **11a**,**b**

Additionally, the CD spectra of the 30mer oligonucleotides were recorded. All spectra showed a maximum at 280–290 nm and a minimum at 240–250 nm, which indicates that these oligonucleotides adopt a B-type DNA conformation, no matter if they are damaged by a strong or a borderline carcinogen at the N^2-position or the C8-position.

In summary, we have developed an efficient strategy for the synthesis on N^2-arylamine modified adducts of 2′-deoxyguanosine involving a palladium-catalyzed cross-coupling reaction and for their corresponding phosphoramidites. These were suitable for the site-specific incorporation into oligonucleotides via solid-phase synthesis on a DNA synthesizer. We have studied the properties of these

oligonucleotides containing the lesions concerning their T_m value and their conformation (CD spectra). Our approach allows now further biochemical studies with regard to the impact of the lesions on replication and repair. These studies are currently under way in our laboratories.

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(23) **General Procedure for the Synthesis of the N^2-Arylhydrazino Adducts (7a,b)**
Under nitrogen atmosphere **5** (1 equiv), racemic BINAP (30 mol%), Pd_2dba_3 (10 mol%), K_3PO_4 (2 equiv), triethylammonium chloride (0.1 equiv), and arylhydrazine (2 equiv) were suspended in dry DME and stirred at 80 °C. After the reaction was completed, sat. $NaHCO_3$ and NaCl solution were added. The layers were separated and the aqueous layer was extracted three times with EtOAc. The combined organic layers were dried over Na_2SO_4 and the solvent was removed in vacuo. The product was isolated by column chromatography using PE–EtOAc (10% → 35%).

(24) Compound **7a**: The general procedure was conducted with 3.05 g of **5** (4.40 mmol) and 1 mL of **6a** (8.8 mmol); yield 2.52 g (3.52 mmol, 80%) of **7a** as a slightly brown solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.46 (s, 2 H, H-8), 7.43–6.82 (m, 9 H, H_{Ar}), 6.67 (dd, $^3J_{H,H}$ = 7.3 Hz, $^3J_{H,H}$ = 7.2 Hz, 1 H, H-1′), 5.34 (s, 2 H, NH), 4.42–4.19 (m, 2 H, H-a), 3.72–3.71 (m, 3 H, H4′, H5′a, H5′b), 3.06 (t, $^3J_{H,H}$ = 6.9 Hz, $^3J_{H,H}$ = 6.9 Hz, 2 H, Hb), 2.97–2.91 (m, 1 H, H2′a), 1.91 (s, 1 H, H2′b), 0.75 [s, 9 H, $Si(CH_3)_3$ at C3′], 0.80 [s, 9 H, $Si(CH_3)_3$ at C5′], 0.00 [s, 6 H, $Si(CH_3)_2$], –0.15 [2 × s, 2 × 3 H, $Si(CH_3)_2$] ppm. ^{13}C NMR (101 MHz, DMSO-d_6): δ = 159.1 (C-6), 156.7 (C-2), 152.4 (C-D), 145.7 (C-A), 144.4 (C-f), 139.6 (C-8), 130.0 (C-C), 129.5 (C-c), 127.6 (C-d), 127.5 (C-e), 123.6 (C-4), 119.0 (C-B), 118.6 (CN), 114.8 (C-5), 87.1 (C-4′), 84.0 (C-1′ 71.6 (C-3′), 67.8 (C-a), 62.8 (C-5′), 39.7 (C-2′), 34.7 [2 × $SiC(CH_3)_3$], 34.3 (C-b) ppm. Mp 84–87 °C; $[\alpha]_{546}^{20}$ –3 (*c* 1.0, $CHCl_3$). IR (KBr): 3744, 3037, 1737, 1695, 1612, 1507, 1110, 838 cm^{-1}. HRMS–FAB: *m/z* calcd: 715.3698; found: 716.3736 [M + H$^+$].
Compound **7b**: The general procedure was conducted with 2.36 g of **5** (3.42 mmol) and 1.42 g of **6b** (7.60 mmol); yield 1.35 g (1.71 mmol, 50%) of **7b** as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.19 (s, 1 H, H8), 7.77–7.33 (m, 13 H, H_{ar}), 6.31 (dd, $^3J_{H,H}$ = 6.7 Hz, 1 H, H1′), 5.37, 5.20 (2 × s, 2 × 1 H, NH), 4.70 (t, $^3J_{H,H}$ = 6.9 Hz, 2 H, Ha), 4.54–4.51 (m, 2 H, H3′), 3.85–3.82 (m, 1 H, H4′), 3.72–3.62 (m, 2 H, H5′a, H5′b), 3.17 (t, $^3J_{H,H}$ = 6.9 Hz, 2 H, Hb), 2.97–2.90 (m, 1 H, H2′a), 2.30–2.24 (m, 1 H, H2′b), 0.88, 0.84 [2 × s, 2 × 9 H, $Si(CH_3)_3$], 0.10, 0.00 [2 × s, 2 × 6 H, $Si(CH_3)_2$] ppm. ^{13}C NMR (101 MHz, DMSO-d_6): δ = 159.2 (C6), 157.5, 153.1 (C4), 148.2 (C2), 144.1, 139.7 (C8), 132.0, 128.7, 126.1, 118.7 (CN), 115.1 (C5), 87.1 (C4′), 83.2 (C1′), 72.3 (C3′), 65.8 (Ca), 62.8 (C5′ 38.3 (C2′), 34.1 (Cb), 25.6, 25.5 [2 × $SiC(CH_3)_3$], –5.6, –5.1 [2 × $Si(CH_3)_2$] ppm. Mp 75–78 °C; $[\alpha]_{546}^{20}$ +25 (*c* 0.4, $CHCl_3$). IR (KBr): 2954, 2928, 2857, 1687, 1109, 776, 740, 482 cm^{-1}. MS (HRFAB): *m/z* calcd: 791.4011; found: 791.4035 [M + H]$^+$.

(25) Compound **10a**: ^{1}H NMR (400 MHz, benzene-d_6): δ = 7.84 [s, 1 H, H-8 (I)], 7.81 [s, 1 H, H-8 (II)], 7.71–7.41 [m, 44 H, H_{Ar} (I + II), DMTr-H (I + II)], 6.25 [dd, $^3J_{H,H}$ = 6.4 Hz, $^3J_{H,H}$ = 6.3 Hz, 2 H, H-1′ (I + II)], 4.74–4.58 [m, 2 H, NH (I)], 4.47–4.37 [m, 2 H, NH (II)], 4.16–4.05 [m, 4 H, H-a (I + II)], 3.58–3.35 (m, 12 H, OCH_3, H-5′a/b (I + II), H-b(I)] 3.15–3.02 [m, 2 H, H-b (II)], 2.54–2.29 (m, 12 H, *i*-PrH (I + II), H-α (I + II), H-2′a/b (I + II)], 1.81 [t, 2 H, H-β (I)], 1.73 [m, 2 H, H-β (II)], 1.15–1.01 [m, 24 H, CH_3*i*-Pr (I + II)] ppm. ^{13}C NMR (101 MHz, benzene-d_6): δ = 160.1, 159.3, 158.7, 148.2, 145.8, 143.3, 138.0, 137.4, 136.1, 132.0, 131.4, 130.6, 130.1, 129.8, 119.0, 113.7, 113.0, 111.0, 87.1, 74.4, 74.2, 66.0, 54.8, 45.3, 43.6, 35,1, 24.7, 24.6, 22.8 ppm. ^{31}P NMR (162 MHz, benzene-d_6): δ = 149.35, 149.19 ppm. Mp 68–71 °C. $[\alpha]_{546}^{20}$ +183 (*c* 1.0, $CHCl_3$). IR KBr): 3870, 3816, 3752, 3744, 3676, 3447, 2966, 1654, 1609, 1581, 1508, 1465, 1383, 1250, 1179, 1034, 829 cm^{-1}. MS–FAB: *m/z* calcd: 989.4353; found: 990.5 [M + H]$^+$.
Compound **11a**: ^{1}H NMR (400 MHz, benzene-d_6): δ = 7.88 [s, 1 H, H-8 (I)], 7.85 [s, 1 H, H-8 (II)], 7.62–6.58 [m, 49 H, DMTr-H (I + II), H_{Ar} (I + II)], 6.27–6.18 [m, 2 H, H-1′ (I + II)], 5.07–4.95 [m, 2 H, H-3′ (I + II)], 4.73–4.67 [m, 4 H, H-a (I + II)], 4.13–4.06 [m, 2 H, H-4′ (I + II)], 3.54–3.26 [m, 20

H, OCH_3 (I + II), H-b (I + II), H-5′ (I + II)], 3.00–2.82 [m, 2 H, H-2′a (I + II)], 2.72–2.61 [m, 8 H, *i*-PrH (I + II), H-a (I + II)], 2.56–2.47 [m, 1 H, H-2′b (I)], 2.41–2.29 [m, 1 H, H-2′b (II)], 1.73–1.67 (m, 4 H, H-b (I + II)], 1.13–1.05 [m, 24 H, CH_3*i*-Pr (I + II)] ppm. ^{13}C NMR (101 MHz, benzene-d_6): δ = 190.6, 186.5, 185.4, 177.7, 169.4, 168.0, 163.6, 159.3, 153.4, 146.1, 145.8, 143.3, 136.3, 132.1, 130.7, 129.9, 129.4, 124.0, 113.8, 87.6, 87.0, 54.8, 47.5, 43.6, 35.2, 24.6 ppm. ^{31}P NMR (162 MHz, benzene-d_6): δ = 149.46, 149.16 ppm. Mp 86–89 °C; $[a]_{546}^{20}$ +39 (*c* 1.0, $CHCl_3$). IR (KBr): 3854, 3744, 3676, 3432, 2965, 2227, 1607, 1508, 1445, 1341, 1251, 1178, 1034 cm^{-1}. MS–FAB: *m*/*z* calcd: 987.4197; found: 988.7 $[M + H]^+$.

Compound **10b**: ^{1}H NMR (400 MHz, benzene-d_6): δ = 7.88 [s, 1 H, H8 (I)], 7.86 [s, 1 H, H8 (II)], 7.62–6.58 [m, 52 H, DMTr-H (I + II), H_{ar} (I + II)], 6.29–6.19 [m, 2 H, H1′ (I + II)], 5.76, 5.37 [2 × s, 2 × 2 H, NH (I + II)], 5.04–5.01 [m, 2 H, H3′ (I + II)], 4.60–4.50 [m, 4 H, Ha (I + II)], 4.13–4.08 [m, 2 H, H4′ (I + II)], 3.52–3.17 [m, 20 H, OCH_3 (I + II), Hb (I + II), H5′ (I + II)], 2.98–2.93 [m, 1 H, H2′a (I)], 2.89–2.84 [m, 1 H, H2′a (II)], 2.71–2.68 [m, 8 H, *i*-PrH (I + II) + a′ (I + II)], 2.56–2.48 [m, 1 H, H2′b (I)], 2.41–2.34 (m, 1 H, H2′b (II)], 1.73–1.67 [m, 4 H, b′ (I + II)], 1.13–1.05 [m, 24 H, CH_3*i*-Pr (I + II)] ppm. ^{13}C NMR (101 MHz, benzene-d_6): δ = 159.1, 155.6, 153.2, 144.1, 139.7, 135.6, 134.6, 130.0, 127.8, 122.3, 118.8, 116.2, 109.1, 87.6, 83.1, 70.8, 65.8, 61.7, 39.7, 34.4 ppm. ^{31}P NMR (162 MHz, benzene-d_6): δ = 149.87, 149.09 ppm. Mp 75–78 °C; $[a]_{546}^{20}$ +33 (*c* 0.1, $CHCl_3$). IR (KBr): 3744, 3675, 2966, 1607, 1457, 1200, 1077, 978 cm^{-1}. MS–FAB: *m*/*z* calcd 1065.4666; found: 1066.6 $[M + H]^+$.

Compound **11b**: ^{1}H NMR (400 MHz, benzene-d_6): δ = 7.88 [s, 1 H, H8 (I)], 7.86 [s, 1 H, H8 (II)], 7.62–6.58 [m, 59 H, DMTr-H (I + II), H_{ar} (I + II)], 6.29–6.19 [m, 2 H, H1′ (I + II)], 5.04–5.01 (m, 2 H, H3′ (I + II)), 4.60–4.50 [m, 4 H, Ha (I + II)], 4.13–4.08 [m, 2 H, H4′ (I + II)], 3.52–3.17 [m, 20 H, OCH_3 (I + II), Hb (I + II), H5′ (I + II)], 2.98–2.93 [m, 1 H, H2′a (I)], 2.89–2.84 [m, 1 H, H2′a (II)], 2.71–2.68 [m, 8 H, *i*-PrH (I + II) + α′ (I + II)], 2.56–2.48 [m, 1 H, H2′b(I)], 2.41–2.34 [m, 1 H, H2′b(II)], 1.73–1.67 [m, 4 H, β′ (I + II)], 1.13–1.05 [m, 24 H, CH_3*i*-Pr (I + II)] ppm. ^{13}C NMR (101 MHz, benzene-d_6): 159.1, 155.6, 153.2, 144.1, 139.7, 135.6, 134.6, 130.0, 127.8, 122.3, 118.8, 116.2, 109.1, 87.6, 83.1, 70.8, 65.8, 61.7, 39.7, 34.4 ppm. ^{31}P NMR (162 MHz, benzol-d_6): δ = 149.29, 149.08 ppm. Mp 63–65 °C; $[\alpha]_{546}^{20}$ –13 (*c* 0.4, $CHCl_3$). IR (KBr): 3821, 3675, 3447, 2966, 1734, 1700, 1653, 1507, 1363, 1179, 1033, 829 cm^{-1}. MS–FAB: *m*/*z* calcd: 1063.4510; found: 1064.7 $[M + H]^+$.