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Analytical studies of 'mixed sequence' oligodeoxyribonucleotides synthesized by competitive coupling of either methyl- or β-cyanoethyl-*N*,*N*-diisopropylamino phosphoramidite reagents, including 2'-deoxyinosine

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ABSTRACT

High-performance liquid chromatography (HPLC) and $^{1}H/^{31}P$ nuclear magnetic resonance (NMR) spectroscopy were used to measure the molar ratio of oligodeoxyribonucleotide products in mixtures obtained with automated DNA synthesizers that employed competitive coupling of either standard methyl- or newer β-cyanoethyl-N,N-diisopropylamino phosphoramidite reagents, which include deoxyinosine. Mixtures of these reagents when used as freshly prepared solutions afforded ratios of products that indicated negligibly small differences among the rates of the various competitive coupling reactions. However, studies of reagent stability in solution revealed that both types of the N-isobutyryl deoxyguanosine reagent decompose faster than their corresponding dA, dC, and dT phosphoramidites, which led to significantly lower proportions of dG-containing sequences. This problem was attenuated for the β-cyanoethyl reagents due to their slower rate of decomposition.

INTRODUCTION

Mixtures of synthetic oligodeoxyribonucleotides which represent all possible coding sequences for a particular peptide fragment are useful as probes for nucleic acids, and as primers for obtaining chain extension products and sequence information.[1,2] The original approach for synthesizing these "mixed sequence" oligonucleotides employed the phosphotriester method and mixtures of trinucleotide coupling units that had a different base at the central position.[3] This strategy, which affords approximately equimolar amounts of the desired sequences,[4] has been successfully applied to the use of mixtures of either dimeric or monomeric coupling units, as judged from analyses of products using HPLC[5] and DNA sequencing.[5,6] While these analytical data and reported[1,7] applications of phosphotriester-derived mixed sequence probes support the reliability of the aforementioned synthetic methods, it does not follow that new types of coupling reagents[8-16] will likewise afford functional (near-equimolar) distributions of mixed sequence products.

Analytical data supportive of competitive coupling of deoxynucleoside methylphosphochloridites was reported[15] several years ago; however, these

reagents did not become as popular as the subsequently introduced[16] methyl-N,N-diisopropyl (iPr_2) phosphoramidites ("methyl amidites"). Our initial evidence for nonselective coupling of T vs. C methyl amidites was provided by successful use of 32-and 64-fold redundant 18-mer probes.[17] By contrast, competitive coupling of A vs. G methyl amidites to obtain a relatively simple 8-fold redundant 17-mer probe repeatedly gave material which failed to detectably hybridize with cloned DNA, by comparison to formally the same mixture of probes that were synthesized[18] by the phosphotriester method using dimer couplings.[5] Since perfect matching with the target sequence required G at two of the three A-or-G positions in this 17-mer probe, we considered the possibility of inadequate reaction of G methyl amidite in its competitive couplings. The difficulty of sequence analysis of mixed probes[6,14] led us to investigate shorter mixed sequence oligomers as tractable products that could be readily separated and quantified by HPLC.[5,15] Toward the end of these studies we included the newly introduced β-cyanoethyl-N,N-iPr_2 phosphoramidites,[13,14] which have attracted considerable attention. Single sequence alternatives to mixed sequence probes/primers have also been recently investigated.[19-21] Promising results[21a] with deoxyinosine-containing single sequences prompted our study of competitive coupling of I vs. C using either methyl or β-cyanoethyl amidite reagents as an alternative to quaternary mixtures of A, G, C and T.

RESULTS AND DISCUSSION

Early Studies of Methyl-N,N-diisopropyl Phosphoramidites

Table I contains analytical data for HPLC-separated 5'-dimethoxytrityl ("DMT") products; integrated peak areas measured at 254 nm were corrected for differences in molecular absorptivity. Runs 1-5 employed deoxynucleoside-3'-O-succinates bonded to aminopropyl-derivatized silica,[10] and two different lots of preweighed "equimolar" mixtures of A and G methyl amidites diluted to a total, nominal concentration of 0.2 M, i.e., 0.1M A and 0.1M G. The roughly 2:1 ratio of incorporated A:G residues indicated that there may have been a "bias" against the coupling of G, and that this effect was essentially independent of the chainlength and base composition of the 5'-HO acceptor. Individual solutions of A and G amidites were then used to prepare a mixture that was nominally 0.067M A and 0.13M G. The data obtained for runs 6-10 employing this 1:2 mixture of A:G amidites clearly indicated that compensation for the "bias" was possible; however, the origin of the "bias" was not apparent.

TABLE I. Mixed-sequence 5'-DMT products from methyl phosphoramidites

run no.	product	conditions[b]	molar ratio of products[a] A	G	C	T
1	(A/G)TT	0.1M, A+G lot 1	66.7	33.3	-	-
2	(A/G)TT	0.1M, A+G lot 2	69.1	30.9	-	-
3	(A/G)TTTTT	"	70.2	29.8	-	-
4	(A/G)GG	"	61.7	38.3	-	-
5	(A/G)AA	"	68.8	31.2	-	-
6	(A/G)TT	0.067M A,0.133M G	50.0	50.0	-	-
7	(A/G)TTTTTTTT	"	47.9	52.1	-	-
8	(A/G)AA	"	52.6	47.4	-	-
9	(A/G)AAAAA	"	51.9	48.1	-	-
10	(A/G)CC	"	54.3	45.7	-	-
11a	(C/T)TT	0.1M, C+T lot 3	-	-	50.5	49.5
11b	(C/T)TT	"	-	-	50.5	49.5
12	(C/T)TTTTT	0.1M, C+T lot 4	-	-	46.8	53.2
13	(C/T)GG	0.1M, C+T lot 3	-	-	44.4	55.6
14	(C/T)AA	"	-	-	48.7	51.3
15a	(N)TT	0.05M each, lot 5	32.0	18.0	25.0	25.0
15b	(N)TT	"	32.0	18.0	25.0	25.0
16	(N)TTTTT	"	35.4	12.5	24.0	28.1
17	(N)TTTTTTTT	"	35.2	12.5	22.4	26.8

[a]HPLC column A, gradient I. [b] Nominal concentration of each amidite in the mixed coupling step, based on unconfirmed specifications of the manufacturer (vendor A) for prepackaged material. Molar ratio tetrazole:total amidite: 5'-HO acceptor ≅ 50:20:1.

The procedure described above for runs 1-5 was applied to C and T reagents in runs 11-14, which demonstrated that these competitive coupling reactions of C and T were essentially independent of the chainlength and base composition of the 5'-HO acceptor, and gave near-equimolar mixtures of products, i.e., there was no apparent "bias" like that found for A and G. The same procedure was used in runs 15-17 to study A, G, C, and T ("N") at a total concentration of amidite that was nominally 0.2 M. In runs 16 and 17, all four of the respective 6-mer and 9-mer 5'-DMT products were separated by HPLC, which revealed that the ratios of products were essentially equal to a composite of the data described above for A and G (runs 1-5) and C and T (runs 11-14). Similar findings were obtained in duplicate runs 15a and 15b, which required detritylation of the crude product mixture and separation of the

5'-HO species.

Although these early results summarized in Table I indicated clearly that there was a ca. 2-fold difference between the apparent coupling efficiency of the A and G methyl amidites, the magnitude of this effect was unprecedented in studies of phosphotriester[5] and phosphochloridite[15] reagents, and subsequent independent measurements showed no significant difference in reactivity among the A and G methyl amidites.[22] We therefore undertook a more detailed investigation of this subject. Before describing this work, a number of technical points are worth noting: (1.) Methyl amidites were obtained from two manufacturers as numerous different lots over a period of one year; samples of β-cyanoethyl amidites were available from only one manufacturer. (2.) To avoid uptake of water during weighings, solutions of mixtures of the amidites were prepared by combining aliquots of stock solutions. (3.) The aminopropyl-derivatized silica was replaced with alkylamine-derivatized controlled pore glass,[10] which had improved performance characteristics and is now widely employed. (4.) Our single-column DNA synthesizer, which was a prototype model from Applied Biosystems, Inc. (ABI), was replaced with an ABI three-column synthesizer. The ability to perform three syntheses in parallel allowed for more controlled comparisons of competitive coupling reactions and higher precision for replicate runs.

NMR Measurement of Competitive Coupling of Methyl- and β-Cyanoethyl-N,N-diisopropyl Phosphoramidites

It was desirable to analyze the composition of mixed sequence oligonucleotides by HPLC and NMR spectroscopy to check, unambiguously, the accuracy of our estimated molar extinction coefficients. This validation procedure was first applied to a mixture of CTT and TTT or, more briefly, (C/T)TT, which was synthesized (3 x 1 μmol) using a freshly prepared solution of C (0.2 M) and T (0.2 M) methyl amidites. Triplicate HPLC analyses of the crude 5'-DMT (C/T)TT products gave an estimated molar ratio of 49.5:51.5 (± 0.3) for 5'-DMT CTT to 5'-DMT TTT. Co-collection of these products by HPLC followed by detritylation afforded (C/T)TT. Triplicate HPLC analyses of this mixture (Table II, run 18) gave an estimated molar ratio of 49.3:51.7 (±0.3) for CTT to TTT, and thus indicated that the HPLC isolation and detritylation procedures did not alter the composition of the mixture of products. Comparison of ^{1}H NMR spectra of authentic (independently synthesized) CTT, authentic TTT, and this sample of (C/T)TT showed (Figure 1A) non-overlapping signals for the C H-6 proton (d, 7.5 Hz, 7.88 ppm) in CTT, and one of the T H-6 protons (s, 7.72 ppm) in TTT, the integrated intensities of

TABLE II. Mixed-sequence 5'-HO products from methyl phosphoramidites

run no.	products	conditions[b]	molar ratio of products[a] A	G	C	T
18	(C/T)TT	0.2M, vendor A, lot 6	-	-	49.3	51.7
19	(C/T)CC	"	-	-	48.6	51.4
20	(A/G)TT	0.2M, vendor A, lot 7	47.4	52.6	-	-
21	T(A/G)T	0.1M, vendor B, day-0	49.0	51.0	-	-
22	T(A/G)T	day-7	67.8	32.2	-	-
23	T(A/G)T	day-14	79.7	20.3	-	-
24	T(N)T	day-0	24.0	25.1	24.5	26.4
25	T(N)T	day-7	26.6	19.3	23.2	30.9
26	T(N)T	day-14	30.4	10.8	29.0	29.8
27	T(A/G)T	0.1M, vendor A, day-0	48.0	52.0	-	-
28	T(A/G)T	day-2	63.0	37.0	-	-
29	T(N)T	day-0	23.5	29.0	23.2	24.3
30	T(N)T	day-2	25.8	20.5	26.7	27.0
31	T(N)T	day-7	26.3	13.4	28.9	31.4
32	T(N)T	0.05M,vendor A, day-0	27.4	18.3	27.0	27.3
33	T(N)T	" day-6	28.6	15.0	27.8	28.6
34	T(N)T	" day-12	30.6	11.1	32.1	26/3
35	T(N)T	" day-18	31.6	7.5	33.2	27.7

[a]HPLC column A, gradient II for runs 18-20; column B, gradient III for runs 21-31.[b] Nominal concentration of each amidite in freshly prepared mixtures (except in runs 18-20 and 32-35 with prepackaged material) using various different lot nos. Initial molar ratio tetrazole:total amidite:5'-HO acceptor ≅ 50:40:1 (runs 18-20, 24-26, 29-31) or 50:20:1 (runs 21-23, 27, 28) or 50:10:1 (runs 32-35).

which gave 49:51 (±2) as the true molar ratio of CTT to TTT in the mixture of (C/T)TT products, and agreed quite well with the estimated molar ratio of 49.3:51.7 derived from HPLC. The mixture of C and T methyl amidites used in run 18 was also employed in the synthesis of 5'-DMT (C/T)CC, which was collected by HPLC and detritylated to afford 5'-HO (C/T)CC (run 19). There was excellent agreement between the HPLC-derived, near-equimolar ratio of

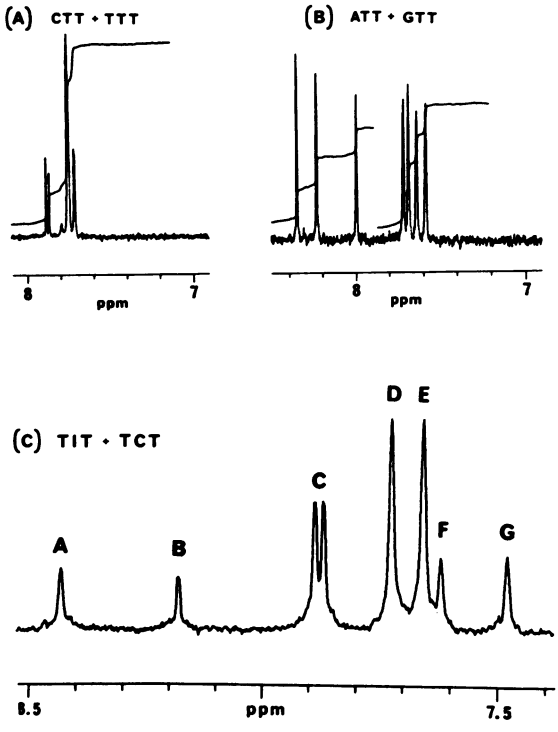

Figure 1. Partial ^{1}H NMR spectra (400 MHz, D_2O) of mixed sequence trimers (C/T)TT (trace A), (A/G)TT (trace B), and T (I/C)T (trace C).

CCC:TCC products found as the 5'-DMT (48.7:51.3) and 5'-HO (48.6:51.4) species.

A mixture of A and G methyl amidites (0.2 M each) was similarly used to synthesize (3 x 1 μmol) the mixture of (A/G)TT trimers. HPLC analysis (run 20) gave 47.4:52.6 (± 0.6) for ATT:GTT, and 50.1:49.9 (± 0.5) for the corresponding 5'-DMT precursors, or an average of ca. 49:51 for A:G, which differed markedly from the earlier ratio of ca. 70:30 (Table I, runs 1 and 2). Comparison of ^{1}H NMR spectra recorded for authentic ATT, authentic GTT, and the (A/G)TT products from run 20 showed (Figure 1B) non-overlapping signals for all of the vinyl-type protons; in ATT: A H-2 (8.35 ppm) and A H-8 (8.25 ppm) plus two T H-6 (7.68 and 7.58 ppm); in GTT: G H-8 (8.00 ppm) plus two T H-6 (7.72 and 7.63 ppm). The integrated signal intensities for these adenyl and guanyl protons gave a molar ratio of 52:48 (± 2) for ATT to GTT, while the integrated signal intensities for the two sets of thymine protons gave an independently determined but essentially equivalent molar ratio of 51:49 (± 2)

for ATT to GTT. It was concluded that our HPLC compositional analysis of oligonucleotide mixtures was accurate and, moreover, that our earlier findings (Table I), which had indicated a "bias" against the competitive coupling of G methyl amidite, did not result from inherent differences in the rates of the competitive coupling reactions that were studied.

Assuming that deoxyinosine (I) forms base pairs with A, C, or T,[21a] but not G, the competitive coupling of I vs. C as a "pseudo 4-fold redundant" substitute for A/G/C/T represents a strategy for reducing the number of sequences in a mixed probe. The mixed sequence trimer T(I/C)T was synthesized (3 x 1 μmol) using a 53:47 molar ratio of I (0.11 M) to C (0.097 M) β-cyanoethyl-N,N-iPr$_2$ phosphoramidites, and then resynthesized (3 x 1 μmol) using a 22:78 molar ratio of methyl-N,N-iPr$_2$ I phosphoramidite (0.11 M) to β-cyanoethyl-N,N-iPr$_2$ C phosphoramidite (0.38 M). We were unable to achieve adequate HPLC separation of the TIT and TCT products; however, the two synthetic mixtures afforded ^{1}H NMR spectra with different ratios of signal intensities that led to unambiguous signal assignments as shown in Figure 1C. For TIT there were characteristic singlets for I H-2 (8.44 ppm, A) and H-8 (8.19 ppm, B, D_2O-exchangeable) plus two singlets for T H-6 (7.62 and 7.48 ppm, F and G); TCT featured a doublet for C H-6 (7.5 Hz, 7.88 ppm, C) and two singlets for T H-6 (7.73 and 7.67 ppm, D and E). The 53:47 mixture of I:C β-cyanoethyl amidites gave a 57:43 mixture of TIT:TCT, which demonstrated essentially nonselective competitive coupling of this pair of reagents. The 22:78 mixture of I methyl-amidite to C β-cyanoethyl-amidite gave a 24:76 mixture of TIT:TCT, likewise demonstrating essentially nonselective competitive coupling.

NMR Studies of the Decomposition of Phosphoramidite Reagents

The relative rate of decomposition of G amidite was studied by ^{31}P NMR using a mixture of G and A methyl amidites to avoid artifacts caused by differences in reaction conditions. It was found (data not shown) that a freshly prepared, nominally equimolar solution of G (0.05 M) and A (0.05 M) methyl amidites in "dry" acetonitrile did in fact contain close to equal amounts of the G and A reagents, and that both pairs of diastereomers underwent relatively slow formal "hydrolysis" at the same rate (ca. 6% reaction after 72 h, 20°C) to give phosphonates, as the observable end-products, which were seen as upfield signals.[16] This reactivity pattern was not detectably influenced by addition of water (0.5% v/v) to the acetonitrile solution. Interestingly, the subsequent addition of 15 mol-% tetrazole, the coupling catalyst,[23] led to accelerated decomposition (ca. 50%

total reaction after 15 h, 20°C) wherein the G phosphonates were formed faster than the A phosphonates by a factor of roughly 2, based on the initial rates of product appearance over a 120-min period. However, the kinetics did not appear to obey a simple pseudo-first-order rate law (leveling-off effect after 6-8 h), and confirmatory data derived from the initial rates of starting material disappearance could not be obtained due to overlapping peaks. For comparative purposes, the tetrazole-catalyzed decomposition of the G and A β-cyanoethyl amidites was similarly studied. It was found (data not shown) that while the diastereomers of A had isochronous signals, this apparent single resonance absorption was flanked by the well-resolved signals due to the diastereomers of G. The initial rates of starting material disappearance over a 120-min period indicated that tetrazole-catalyzed decomposition to phosphonates occurred faster for G, relative to A, by a factor of roughly 1.3. Thus, for example, the ratio of the A:G β-cyanoethyl amidites changed from an initial value of *ca.* 50:50 to a value of *ca.* 70:30 after 6 h of exposure to water (0.5% v/v) and tetrazole (15 mol-%) in acetonitrile.

The aforementioned NMR spectra recorded for G and A amidites, either in the presence or absence of tetrazole, exhibited relatively low intensity signals *ca.* 10 ppm upfield from those of the starting materials, which could be due to phosphitylation of the O^6-position of the G reagent, based on analogy to reported[24,25] signal assignments. The occurrence of this phosphitylation reaction under the present conditions, while purely speculative at this point, offers one possible rationale of the data if one assumes that the reported[25] "instability of the phosphite linkage" in the derived adducts leads to formation of the accumulated phosphonates seen by NMR. Further studies are needed to unambiguously differentiate among this and other conceivable mechanisms which can account for the moderately selective, tetrazole-catalyzed decomposition of G amidite, but essentially nonselective, tetrazole-catalyzed, competitive coupling of G during syntheses of mixed sequence oligonucleotides.

The advantages offered by phosphoramidites having increased stability in solution prompted our comparison of methyl amidites with their newer β-cyanoethyl counterparts. Phosphorus NMR spectroscopy was used to measure the relative rates of tetrazole-catalyzed hydrolysis and, as detailed in Figure 2, it was found that both of the diastereomers of the β-cyanoethyl T amidite underwent hydrolysis *ca.* 6-times slower than their methyl counterparts. This relative reactivity was confirmed by the following experiments.

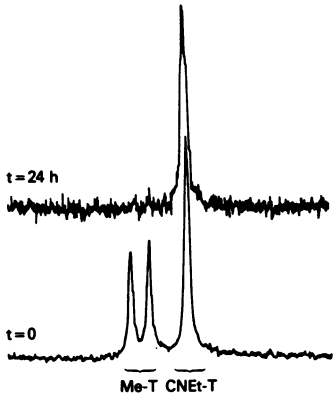

Figure 2. Partial ^{31}P NMR spectra (36 MHz, CH_3CN) of 0.05 M methyl- ("Me-T", 146.2 and 145.9 ppm) and β-cyanoethyl-N,N-diisopropylamino phosphoramidites ("CNEt-T", 143.5 ppm) in the presence of 0.5% v/v water and 15 mol-% tetrazole after mixing (t=0) and one day later (t=24 h). Based on initial rates of disappearance, half-life = 17 min and 18 min for Me-T at 146.2 and 145.9 ppm, respectively, and half-life = ca. 106 min for both diastereomers of CNEt-T.

Time-Course Stability Studies with Methyl-N,N-diisopropyl Phosphoramidites

For the purpose of comparing our results with data obtained independently by others,[22] who chose the mixed sequence trimers T(C/T)T, T(A/G)T, and T(A/G/C/T)T as test cases, we conducted time-course studies of reagent stability using the synthesis of T(A/G)T and "TNT" (N = A/G/C/T), which focused on the behavior of the G reagent and used mixtures of amidites that were kept connected to the synthesizer at ambient room temperature. Prior to each synthesis a procedure was used to displace solutions of amidites and tetrazole which had resided in "exposed" plastic delivery lines leading from the reservoirs of these reagents to a manifold of valve ports. That this displacement or line-purging process is critical for expulsion of hydrolyzed reagent and "wet" acetonitrile, and obtainment of high-yield couplings plus acceptable mixed sequence distributions, was evidenced by data for syntheses of TNT without and with the purging process, using a commercially available "equimolar" mixture of A, G, C, and T methyl amidites at a total concentration of 0.1 M, as recommended by the supplier. This mixed reagent, immediately following dissolution, was found by HPLC (Figure 3, trace A) to give relative ratios of A:G:C:T = 27.4:18.3:27.0:27.3 in the competitive coupling step, which proceeded with a 96% yield (i.e., 4% residual dT was detected). Repetition of the synthesis after a 4-day period and without line-purging gave (Figure 3, trace B) A:G:C:T = 33.4:8.6:31.0:27.0 in the competitive coupling step that now occurred with only a 48% yield. Use of in-line reagent N for this synthesis was equivalent to performing the line-purging process; this was followed by a third preparation of TNT that was found (Figure 3, trace C) to give A:G:C:T = 28.6:15.0:27.8:28.6 in the competitive coupling step, with a 96% yield. In addition to revealing that the freshly prepared mixed reagent

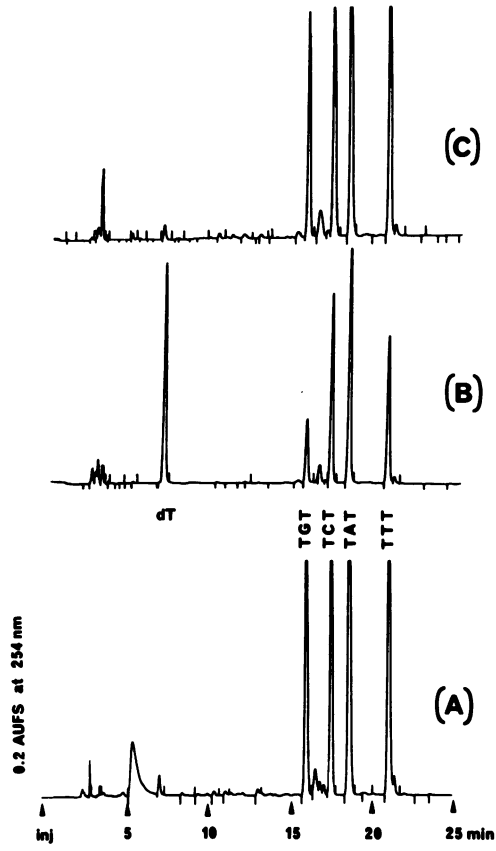

Figure 3. HPLC (column B, gradient III) traces of crude mixtures of 5'-HO TGT (15.9 min), TCT (17.4 min), TAT (18.5 min), and TTT (20.9 min) obtained from competitive coupling using a commercially available "equimolar" mixture of A, G, C, and T methyl amidites immediately after dissolution (trace A), 4 d later without "purging" (trace B, dT at 7.0 min), and on the fifth day, with "purging" (trace C). See text for details.

gave only 73% of the theoretical proportion of TGT (18.3 vs. 25 mol-%), it was evident from these findings that the rate of decomposition of the G methyl amidite was fast by comparison to the corresponding A, C, and T reagents, which was subsequently supported by NMR measurements (vide infra). While this decomposition must have been largely localized to delivery lines external to the reagent reservoir (cf. Figure 3, trace C vs. trace B), the onset of detectable levels of reagent decomposition in the reservoir of G methyl amidite was suggested by the decreased proportion of TGT product after 4 d (18.3 vs. 15 mol-%). This was clearly evidenced by later time-points, as

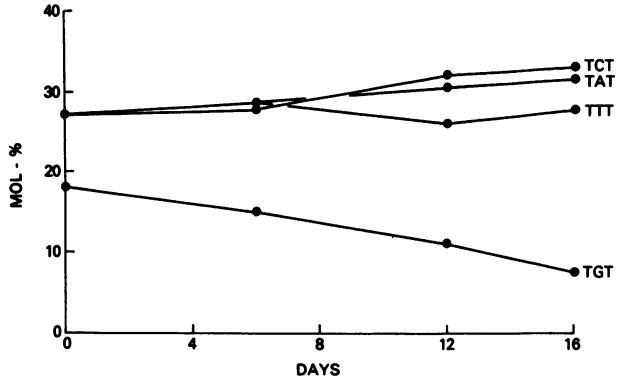

Figure 4. Time-course for the molar composition of mixed sequence trimer products derived from competitive coupling starting with a commercially available "equimolar" mixture of A, G, C, and T methyl amidites. Initial molar ratio of tetrazole : total amidite : 5'-HO acceptor (dT) = ca. 50:10:1. See Table II runs 32-35 for listing of numerical values. Mixed coupling step-yields = 96% on day-0, 96% on day-6, 82% on day-12, and 51% on day-16.

shown in Figure 4, and the following experiments.

Aliquots of fresh stock solutions of each methyl amidite were combined to obtain mixtures that were nominally 0.1 M in each reagent. The results of a time-course study for the synthesis of T(A/G)T (Table II, runs 21-23) revealed that the molar ratio of TAT:TGT products changed from 49.0:51.0, at the start of this stability study, to 67.8:32.2 after 7 d; on the other hand, there was no accompanying change in the mixed coupling step-yield (>95%). This ratio of products underwent further change to 79.7:20.3 after a total of 14 d, and the mixed coupling step-yield decreased to 60%. A parallel time-course study conducted with a mixture of all four methyl amidites gave analogous results (runs 24-26). The relative instability of G methyl amidite was also confirmed by independent time-course studies using another manufacturer's reagents to prepare mixtures of A/G (runs 27 and 28) and N (runs 29-31).

^{31}P NMR analysis of a 23-day-old mixture of A/G, which had given a product ratio of TAT:TGT = 77.0:23.0 vs. an initial product ratio of 49.0:51.0 on day-0, showed that the relative integrated signal intensities for the "aged" A:G methyl amidites was equal to 65:35 (±5). Due to partial overlap of signals, the apparent difference between the ca. 65:35 ratio of A:G amidites and the 77:23 ratio of TAT:TGT products was not significant.

Time-Course Stability Studies with β-Cyanoethyl-N,N-diisopropyl Phosphoramidites

Preliminary experiments with freshly prepared A/G, C/T, and N mixtures of

TABLE III. Mixed-sequence 5'-HO products from β-cyanoethyl phorphoramidites

run no.	products	conditions[b]	mixed coupling yield, %[c]	molar ratio of products[a] A	G	C	T
36	T(C/T)T	0.1M, day-0	>95	-	-	45.0	55.0
37	T(C/T)T	day-4	>95	-	-	44.4	55.6
38	T(C/T)T	day-11	>95	-	-	51.9	48.1
39	T(C/T)T	day-21	55	-	-	51.7	48.3
40	T(A/G)T	0.1M, day-0	>95	53.7	46.3	-	-
41	T(A/G)T	day-4	>95	49.8	50.2	-	-
42	T(A/G)T	day-11	79	57.4	42.6	-	-
43	T(A/G)T	day-21	40	70.6	29.4	-	-
44	T(A/G/T)T	0.1M, day-0	>95	28.7	34.0	-	37.3
45	T(A/G/T)T	day-4	>95	29.9	32.1	-	37.9
46	T(A/G/T)T	day-11	85	36.8	30.4	-	32.8
47	T(A/G/T)T	day-21	68	42.1	19.0	-	38.9
48	T(N)T	0.1M, day-0	-	29.9	21.3	25.6	23.2
49	T(A/G)T	"	-	49.1	50.9	-	-
50	T(C/T)T	"	-	-	-	38.5	61.5
51	T(N)T	0.1M, day-0	-	18.5	14.7	33.8	32.9
52	T(A/G)T	"	-	59.8	40.2	-	-
53	T(C/T)T	"	-	-	-	55.5	44.5

[a]HPLC column B, gradient III. [b] Nominal concentration of each amidite in various freshly prepared mixtures obtained from the same stock solutions of A, G, C, and T reagents (vendor B). Initial molar ratio tetrazole:amidite:5'-HO acceptor (dT) ≅ 50:20:1 (runs 36-43) or 50:31:1 (runs 44-47). [c] Based on relative molar amount of dT determined by HPLC. [d] Same as footnote b except for the reagent source, vendor C.

β-cyanoethyl amidites (0.1 M in each reagent) gave HPLC-determined ratios of products which indicated approximately equal rates of coupling, *viz.*, TAT:TGT = 46.0:54.0, TCT:TTT = 50.3:49.7, and TAT:TGT:TCT:TTT = 23.2:27.5:25.6: 23.7, respectively. A time-course study of the C/T mixture (Table III, runs 36-39) showed that the ratio of TCT:TTT products was essentially invariant over a 21-d period. That the mixed coupling step-yield was 96% after 11 d but only 55% after 21 d was taken as evidence for nonselective degradation of the C/T

reagents. A parallel study of the A/G reagent mixture (runs 40-43) revealed little change in the product ratio after an 11-d period, although the mixed coupling step-yield had decreased to 79%. Decomposition was more apparent after a 21-d period, which afforded a mixed coupling step-yield of only 40% and a ratio of TAT:TGT = 70.6:29.4, consistent with greater loss of G amidite. A parallel study of the ternary A/G/T reagent mixture (runs 44-47) gave results that were in accord with the above findings for C/T and A/G, as well as those for the quaternary mixture of methyl amidites: (1.) initial, approximately equimolar ratios of products, and (2.) gradual preferential degradation of the G β-cyanoethyl amidite, (3.) which was clearly evident after a 21-d period, as judged by the relatively low proportion of TGT product (run 47, ca. 2-fold less abundant) and the poor yield (68%) for the mixed coupling step.

Competitive Coupling with β-Cyanoethyl-N,N,-diisopropyl Phosphoramidites Using Other Automated DNA Synthesizers

All of our results described up to this point refer to competitive couplings using delivery of a solution of premixed amidites. An alternative approach to achieve these syntheses is to use individual amidite solutions which are metered-out and mixed immediately prior to delivery to the solid support. For comparative purposes, we arranged a limited study of competitive coupling using two other automated synthesizers that employed essentially the same chemistry cycle as our machine but were different in that they utilized β-cyanoethyl amidites which were "mixed-on-the-fly." Runs 48-50 were performed with a Vega synthesizer using freshly prepared solutions of each β-cyanoethyl amidite (0.1 M). Approximately equimolar ratios of products were found for both T(N)T (run 48) and T(A/G)T (run 49), whereas T(C/T)T (run 50) gave a ca. 40:60 ratio for C:T incorporation. Runs 51-53, which were similarly performed with a Biosearch synthesizer, gave an entirely different pattern of results: for T(N)T (run 51) there were significant deviations from the expected 25 mol-% incorporation of each base, A:G:C:T ≅ 18:15:34:33, and for T(A/G)T (run 52) the incorporation of A:G = 60:40, whereas for T(C/T)T (run 53) the incorporation was near-equimolar, C:T ≅ 55:45. The reason(s) for the moderate to large deviations from ideal performance found in runs 48-53 is (are) not known at this time, although inaccurate automated metering of reagents is a likely contributing factor for consideration.

SUMMARY

The present study of the synthesis of mixed sequence oligomers by competitive coupling reactions of either methyl- or β-cyanoethyl-N,N-iPr_2

phosphoramidites, using purchased reagents and a commercially available automated synthesizer, has shown that freshly prepared, nominally equimolar, pre-made mixtures of these amidites afforded product distributions which were nearly equimolar (± ca. 1-5 mol-%). The apparent "bias" against G-coupling found in our early experiments (Table I) was not reproduced, for reasons that are not understood; nevertheless, these early studies with methyl amidites showed clearly that the coupling reaction was essentially independent of the chainlength and base composition of the 5'-HO acceptor. It is reasonable to assume that couplings with the analogous β-cyanoethyl compounds are nonselective in the same sense, and that with either class of reagents there is nonselectivity in syntheses involving mixed coupling reactions at multiple positions in the chain.[14] On the other hand, extrapolations to new coupling reactions are risky. Seemingly minor changes involving, for example, the nature of the base-protecting group, the phosphite alkyl group, the dialkylamino group, and/or the catalyst could lead to selective couplings and, therefore, potential problems due to systematic kinetic-bias against a coupling unit. This bias may be detected by colorimetric methods[26] or by HPLC methods such as those described here and elsewhere;[5,22,27] satisfactory product distributions can be obtained by adjusting ratios of reagents. Much more difficult to contend with is the general problem of differential stability among the reagents, as was evidenced herein by the relative instability of the G amidite in solution. This problem, which obtains for reagents that are either pre-mixed or "mixed-on-the-fly," can be obviated by using only freshly dissolved reagents, although cost considerations make this option unattractive, especially in view of the possibility of in situ generation of equimolar mixtures of phosphoramidites.[24] Such innovations and/or the use of less complex product mixtures,[21] will hopefully add to the functional reliability of mixed sequence oligonucleotides synthesized by the now widely employed phosphoramidite method.

EXPERIMENTAL SECTION

ABI Model 380A (Tables II-III) and prototype (Table I) DNA synthesizers were employed as previously described[28] using amidites and solid supports purchased from ABI (vendor A) or American BioNuclear (vendor B). A prototype Vega Coder 300 and a Biosearch (vendor C) SAM ONE/Series II were used according to the manufacturer's instructions and employed amidites obtained from vendors B and C, respectively. A previously described[29] HPLC system was used with Waters μBondapak® or Nova-Pak® columns (A or B, respectively; 7.8 mm

x 30 cm) and linear gradients of increasing acetonitrile (a) vs. 0.1 M triethylammonium acetate (b): gradient I = 1%/min for 10 min then isocratic, initial a:b = 20:80, flow rate = 4 mL/min; gradient II = 0.125%/min for 20 min, initial a:b = 10:90, flow rate = 5 mL/min; gradient III = 0.333%/min for 30 min, initial a:b = 5:95, flow rate = 3 mL/min. Molar extinction coefficients for 2'-deoxynucleosides were determined at 254 nm using 60-150 μM solutions in 30:70 v/v a:b, and gave the following values for ε: dA, 1.34 x 10^4; dG, 1.43 x 10^4; dC, 0.66 x 10^4; dT, 0.72 x 10^4. Product ε-values were derived from weighted sums of these four constants. NMR spectra were recorded as previously described.[28]

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