



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07H 19/00, C07D 307/06, C12Q 1/68	A1	(11) International Publication Number: WO 97/43298 (43) International Publication Date: 20 November 1997 (20.11.97)
(21) International Application Number: PCT/US97/08307 (22) International Filing Date: 15 May 1997 (15.05.97) (30) Priority Data: 60/017,772 15 May 1996 (15.05.96) US (71) Applicant: RESEARCH CORPORATION TECHNOLOGIES, INC. [US/US]; Suite 600, 101 North Wilmot Road, Tucson, AZ 85711-3335 (US). (72) Inventor: KOOL, Eric, T.; 27 Hoyt Place, Rochester, NY 14610 (US). (74) Agents: DiGIGLIO, Frank, S. et al.; Scully, Scott, Murphy & Presser, 400 Garden City Plaza, Garden City, NY 11530 (US).		(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: NOVEL NUCLEOSIDE ANALOGS WITH POLYCYCLIC AROMATIC GROUPS ATTACHED, METHODS OF SYNTHESIS AND USES THEREFOR		
(57) Abstract <p>The present invention provides fluorescent nucleosides carrying polycyclic aromatic hydrocarbons such as anthracene, phenanthrene, pyrene, stilbene, tetracene or pentacene. The subject nucleosides may be synthesized using a C-glycosidic bond formation method employing organocadium or organozinc derivatives of the aromatic compounds and coupling with a 1-α-chlororibose or deoxyribose synthon. The α-anomers of the coupling reaction may be epimerized to the β-anomers by acid-catalyzed equilibration. The fluorescent nucleosides act as a DNA or RNA base analogs and can be incorporated into nucleic acids. Resultant fluorescently tagged nucleic acids are useful as probes for target nucleic acids in tissues, solutions or immobilized on membranes.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

**NOVEL NUCLEOSIDE ANALOGS WITH
POLYCYCLIC AROMATIC GROUPS ATTACHED,
METHODS OF SYNTHESIS AND USES THEREFOR**

1

This invention was made with United States government support under grant number DAAH-04-93-G-5 0431 awarded by the Army Research Office. The United States government may have certain rights in the invention.

FIELD OF THE INVENTION

10

The present invention provides fluorescent nucleosides carrying polycyclic aromatic hydrocarbons such as anthracene, phenanthrene, pyrene, stilbene, tetracene or pentacene. The subject nucleosides may be synthesized using a C-glycosidic bond formation method employing organocadium or organozinc derivatives of the aromatic compounds and coupling with a 1- α -chlororibose or deoxyribose synthon. The α -anomers of the coupling reaction may be epimerized to the β -anomers by acid-catalyzed equilibration. The fluorescent nucleosides act as DNA or RNA base analogs and can be incorporated into nucleic acids. Resultant fluorescently tagged nucleic acids are useful as probes for target nucleic acids in tissues, solutions or immobilized on membranes.

15
20
25

BACKGROUND OF THE INVENTION

A large number of non-natural analogues of DNA nucleosides have been synthesized in recent years. Changing the structure of the base moiety attached to deoxyribose has been a useful strategy for probing

30

35

1 structure and function in DNA. For example, a number
of base analogues have been used to test the
importance of specific hydrogen bonding interactions
which may be important for function of the natural
nucleic acid bases.¹⁻³ Using this strategy, the
5 importance of hydrogen bonding in stabilizing DNA and
RNA structure, in protein interactions and in the
fidelity of enzymatic DNA and RNA synthesis has been
examined.

10 Modified DNA bases have also been
synthesized with the purpose of serving as reporter
groups in physical and biochemical studies of
structure and function. Examples of reporter groups
which have been attached to DNA bases include biotin⁴
and digoxigenin groups⁵, spin-label groups⁶, and DNA-
15 cleaving moieties⁷. Among the most prominent classes
of reporters used in DNA are fluorescent-tagged DNA
bases which can serve as probes in biophysical and
biochemical studies⁸. In contrast to placement of
such reporter groups at the end of a DNA strand using
20 nonnucleotide linkers, the attachment of a reporter to
a DNA base allows for placement and probing within a
stretch of DNA. Such a strategy has found
considerable practical use in fluorescence-based
automated DNA sequencing.⁹ An alternative approach
25 to the conjugation of a fluorophore to a natural DNA
base is the more direct modification of a DNA base
itself to render it fluorescent. A number of modified
DNA bases with useful fluorescence properties have
been reported recently; among the most widely used

30

35

1 nucleosides of this type are 2-aminopurine¹⁰ and
ethenoadenosine¹¹.

DNA may be fluorescently tagged either enzymatically or synthetically. Enzymatic incorporation is carried out by use of nucleoside triphosphates carrying a given fluorophore. Incorporation into DNA by chemical methods is especially common. Chemical methods of incorporating fluorescent reagents into DNA is done by either of two methods: direct incorporation of a label which has been converted to a phosphoramidite derivative or incorporation of an amine into the oligonucleotide, followed by later derivatization with a fluorophore isothiocyanate or NHS ester derivative.

15 The postsynthesis derivatization of DNA is typically inefficient, and requires steps beyond those of standard DNA synthesis as well as laborious purification steps. Direct incorporation of a label into DNA is attractive because standard DNA synthesis and purification steps are used. However, currently available reagents are quite expensive due to their cost of synthesis.

Another drawback to currently used fluorescent-tagged nucleic acids is the quenching phenomenon which occurs when multiple fluorescent tags are placed near each other. Thus, a nucleic acid with multiple fluorescent tags is often no brighter or even less bright than a nucleic acid with a single fluorescent tag.

In addition, it is commonly observed that a fluorescent tag is quenched by the act of attaching it

1 to DNA. For example, it has been reported (Netzel,
1989 J. Am. Chem. Soc. 111:6966) that pyrene tags
attached to a linker at the end of a DNA strand were
quenched greatly (50-fold) in the DNA. Moreover, on
5 binding a complementary sequence, the emission was
quenched another ten-fold. This effect necessarily
leads to lower sensitivity of detection.

Another limitation of commercially available
fluorescent labels include their rapid photobleaching
characteristics. As in fluorescence microscopy or
10 blot hybridization, the practical brightness of a
label depends on the time of integration of the
emission signal. Fluorescein, for example,
photobleaches quite rapidly in a DNA oligonucleotide,
because of its complex structure (allowing greater
15 reactivity) and because it is exposed to solution
where more reactions occur. In addition, a label such
as fluorescein is typically attached to DNA by
flexible tethers. Measuring protein-DNA binding by
time-resolved fluorescence an isotropy is often
20 problematic since the fluorophore tumbles rapidly on
its flexible chain.

The present invention overcomes many of the
shortcomings associated with labeling nucleic acids.
The present invention allows a pyrene, anthracene,
25 phenanthrene, stilbene, tetracene or pentacene-
derivatized nucleoside to be inserted within a DNA or
RNA strand at any position and remain rigidly stacked
within the helix. Because the fluorescent part of the
label is situated as if it were a DNA base, the
30 fluorescent groups are stacked neatly in the helix,

1 and if placed adjacent to each other, interact with
each other strongly, allowing for intense excimer
emission.

5 The fluorescent nucleoside analogs of the
present invention do not photobleach rapidly, making
the labeled sample much longer lived and allowing the
opportunity for measurements and study over a longer
period. Moreover, since the preferred embodiment of
10 the invention provides for α -linkages to the
fluorescent moiety, quenching by adjacent β -linked DNA
bases is minimized. In addition, the direct
attachment of the fluorescent moiety to the sugar
residue in the nucleotide chain eliminates the
flexible linker typically present in fluorescently
15 labeled nucleic acids. This feature simplifies
measurements such as time-resolved fluorescence
anisotropy.

SUMMARY OF THE INVENTION

20 The present invention provides fluorescent
nucleoside analogs carrying a polycyclic aromatic
hydrocarbon such as anthracene, phenanthrene, pyrene,
stilbene, tetracene or pentacene. The polycyclic
aromatic hydrocarbon is attached to the C1 (1')
25 position of a sugar moiety such as ribose or
deoxyribose by a carbon-carbon bond. The sugar moiety
may be a hexose such as glucose or a pentose such as
arabinose. In one embodiment of the invention, the
aromatic hydrocarbon attached to the C1 carbon of a
30 sugar moiety is phenanthrene. In another embodiment of
the invention, the aromatic hydrocarbon attached to

1 the C1 carbon of a sugar moiety is pyrene. In still
another embodiment of the invention, the aromatic
hydrocarbon attached to the C1 carbon of a sugar
moiety is anthracene. In another embodiment of the
invention, the aromatic hydrocarbon attached to the C1
5 carbon of a sugar moiety is stilbene. In another
embodiment of the invention, the aromatic hydrocarbon
attached to the C1 carbon of a sugar moiety is
tetracene. In another embodiment of the invention,
10 the aromatic hydrocarbon attached to the C1 carbon of
a sugar moiety is pentacene.

The present invention also provides
phosphoramidite derivatives of anthracene,
phenanthrene, pyrene, stilbene, tetracene, or
pentacene-derivatized nucleosides. The
15 phosphoramidite derivatives are useful in the chemical
synthesis of nucleic acids containing phenanthrene,
anthracene, pyrene, stilbene, tetracene, or pentacene-
derivatized nucleosides.

The present invention also provides
20 intermediates useful in the synthesis of the subject
nucleosides and subject phosphoramidite derivatives of
anthracene, phenanthrene, pyrene, stilbene, tetracene
or pentacene-derivatized nucleosides. The
intermediates comprise an adduct of Hoffer's
25 chlorosugar and the corresponding polycyclic aromatic
hydrocarbon.

Another aspect of the invention is directed
to methods for synthesizing phenanthrene, anthracene,
pyrene, stilbene, tetracene or pentacene-derivatized
30 nucleosides and their phosphoramidite derivatives. In

1 another aspect of the invention, methods of preparing
fluorescently-labeled DNA or RNA using the subject
nucleosides and phosphoramidite derivatives are also
provided.

5 Fluorescently tagged nucleic acids such as
DNA and RNA having one or more subject fluorescent
nucleosides incorporated within are also provided by
the present invention.

10 The present invention further provides
methods of detecting a target nucleic acid through
hybridization (such as FISH) of complementary nucleic
acid probes employing one or more of the subject
fluorescent nucleosides incorporated within.

15 Both α and β configurations of the
anthracene, phenanthrene, pyrene, stilbene, tetracene
or pentacene derivatized nucleosides are provided as
well as a method for epimerization of the α -anomers to
 β -anomers.

BRIEF DESCRIPTION OF THE DRAWINGS

20 Figure 1 is an Illustration of qualitative
differences in nuclear Overhauser enhancements
observed for alpha-(left) and beta-anomers(right) of
compounds 1-6. In alpha anomers, irradiation of the
C-2'- β proton gives enhancements in both the C-1' and
25 C-3' protons, while irradiation of the C-2'- α proton
gives little or no enhancement for either. In the
beta-anomers, irradiation of the C-2'- β proton gives
enhancement only in the C-3' proton, while irradiation
of the C-2'- α proton gives enhancement only in the C-
30 1' proton.

1 Figure 2 is an ORTEP drawing of 1-naphthyl
nucleoside 3a from single-crystal x-ray structure.
The structure has the α -anomeric configuration and a
C-3'-exo S-type sugar conformation.

5 Figure 3 shows 400MHz proton NMR spectra for
trinucleotides (sequence T-X-T) containing (a)
pyrenyl, (b) phenanthryl, and (c) naphthyl nucleosides
1-3 at the X position.

10 Figure 4A is a fluorescence emission
spectrum for self-complementary heptamer
oligonucleotides containing pyrene as the C-
nucleosides at the 5'-terminal position. The DNA
sequence is 5'XCGCGCG, where X is 1. Excitation is at
341 nm and solutions contain pH 7.0 PIPES (10 mM),
100mM NaCl, 10mM MgCl₂, and DNA strand concentrations
15 of 0.1 and 0.15 μ M.

 Figure 4B is a fluorescence emission
spectrum for self-complementary heptamer
oligonucleotides containing phenanthrene as the C-
nucleosides at the 5'-terminal position. The DNA
20 sequence is 5'XCGCGCG, where X is 2. Excitation is at
251 nm. Solutions and DNA strand composition are the
same as for Fig. 4A.

 Figure 5 depicts the structural formulas of
the compounds of the present invention. Compounds 1-6
25 as shown are the β forms. Compounds 1a through 6a are
the α forms of the molecules depicted in the Figure.

 Figure 6 schematically depicts a synthetic
preparation of the compounds of the present invention.

30 Figure 7 schematically depicts a synthetic
preparation of the compounds of the present invention.

1 Figure 8 is the emission spectra of oligos
containing 1, 2, 3 and 5 fluoresceins from
commercially available phosphoramidite. Strong
quenching occurs with multiple labels.

5 Figure 9 is the emission spectrum of the
oligonucleotide designated P1 in Figure 13.

Figure 10 is the emission spectrum of the
free pyrene nucleoside designated PO in Figure 13.

10 Figure 11 is the emission spectra of
oligonucleotides designated P3, P5, P3END and P3A in
Figure 13.

Figure 12 is the emission spectra of
oligonucleotides designated P3 and P6 in Figure 13.

15 Figure 13 depicts the structures of
complexes of triplex-forming oligonucleotides with the
complementary target sequence 5'-dGAAAAGAAGA.

Figure 14 is a schematic representation of a
synthetic method for the preparation of the α -pyrene
nucleoside of the present invention.

20 Figure 15 is a schematic representation of a
synthetic method for the preparation of the β -pyrene
nucleoside of the present invention.

Figure 16A is a photograph showing
fluorescent DNAs in four plastic tubes held over a
transilluminator. From right to left, tubes contain
25 fluorescein-labeled oligonucleotides with 1, 2, 3, and
5 fluorescein. The intensity decreases as the number
of fluorescein labels increases.

30 Figure 16B is a photograph showing α -pyrene
-labeled oligonucleotides (aqueous buffer with no
deoxygenation) in six plastic tubes held over a

1 transilluminator. The very left tube has one pyrene
label at 10x the concentration of the other five tubes
and shows the typical blue color of pyrene. The other
five tubes are diluted 10-fold and have 1, 2, 3, 6,
and 10 labels from left to right.

5 Figure 17 is a fluorescence emission spectra
for oligonucleotides having either three consecutive
alpha pyrene residues or three consecutive beta pyrene
residues.

10 Figure 18 shows the nucleotide sequences of
oligonucleotides P1, P2, P3, P5, C6 and C10 where P
stands for a pyrene-derived nucleotide. Relative
emission intensities are listed at the right.

15 Figure 19A is an emission spectra for single
pyrene alpha- and beta-nucleosides at 0.1 μ M in
methanol (excitation 348nm).

20 Figure 19B is an emission spectra for alpha-
pyrene in DNA (aqueous buffer) compared to
pyrenemethylamine·HCl at the same concentration
(intensities for pyrene-DNA are multiplied by 10).

25 Figure 20 shows the effect of alpha- vs.
beta-isomeric structure on fluorescence intensity, as
shown by fluorescence emission spectra of HT3 (three
alpha pyrene nucleosides) as compared to the same
sequence with three beta pyrene nucleosides.
Excitation was at 341nm; band at 390nm is due to Raman
scattering and is present with buffer alone.

Figure 21A shows emission spectra for
sequences HT1-HT3, containing 1-3 pyrenes.

30 Figure 21B shows emission spectra for HT3-
HT7, with 3-7 adjacent pyrenes. Excitation was at

1 341nm with equal DNA strand concentration of 0.1 μ M;
see Table 1 for numerical data.

5 Figure 22 shows adsorption spectra for
oligonucleotides containing 1-7 adjacent alpha-pyrene
labels (sequence HT1-HT7). Measurements are carried
out at equal pyrene concentrations (2 μ M in pyrene),
rather than equal DNA strand concentrations, to show
differences in molar extinction due to grouping
effects.

10 Figure 23 shows the even/odd effect on
excimer fluorescence intensity. Plot of number of
pyrenes vs. relative integrated excimer emission
intensity for 3' end series (\square) (sequences HT1-7) and
for 5' end series (o) (HT3B-6B).

DETAILED DESCRIPTION OF THE INVENTION

15 The present invention provides fluorescent
nucleoside analogs having polycyclic aromatic
hydrocarbons such as anthracene, phenanthrene, pyrene,
stilbene, tetracene, or pentacene attached to a sugar
moiety. The aromatic hydrocarbon groups are attached
20 to the C-1 (1') carbon of the sugar moiety in a
nucleotide or nucleoside and act as DNA or RNA base
analogs. Due to the location of the polycyclic
aromatic hydrocarbon on the sugar moiety, the
nucleoside analogs of the present invention stack
25 neatly in an RNA or DNA helix and do not interfere
with binding properties of surrounding complementary
bases.

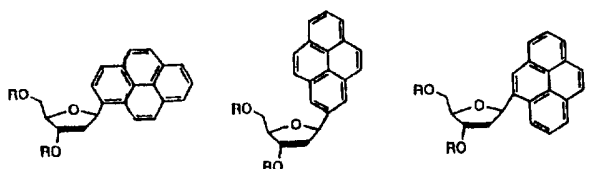
30 In accordance with the present invention,
the anthracene, phenanthrene, pyrene, stilbene,
tetracene, or pentacene may be substituted at various

1 positions on their respective ring structures with one
or more alkoxy, alkylamino or halide groups without
altering the fluorescent properties of the nucleoside
analog. Examples include but are not limited to
5 methoxy, ethoxy, dimethylamino, diethylamino, nitro,
methyl, cyano, carboxy, chloro, bromo, iodo, or amino
groups.

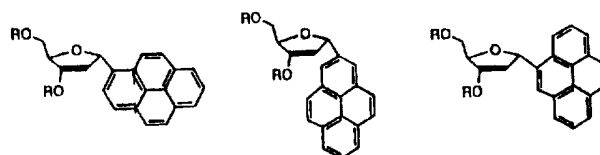
In addition, the anthracene, phenanthrene,
pyrene or other aromatic hydrocarbon may be attached
10 at any available position on their respective ring
structures to the C1 (1') position of a sugar moiety
by a carbon-carbon bond. Both alpha and beta anomers
of the anthracene, phenanthrene, pyrene, stilbene,
tetracene or pentacene-derivatized nucleosides are
provided by the present invention.

15 In one embodiment of the invention, the
pyrene-derivatized deoxynucleoside has at least one of
the following structures:

20



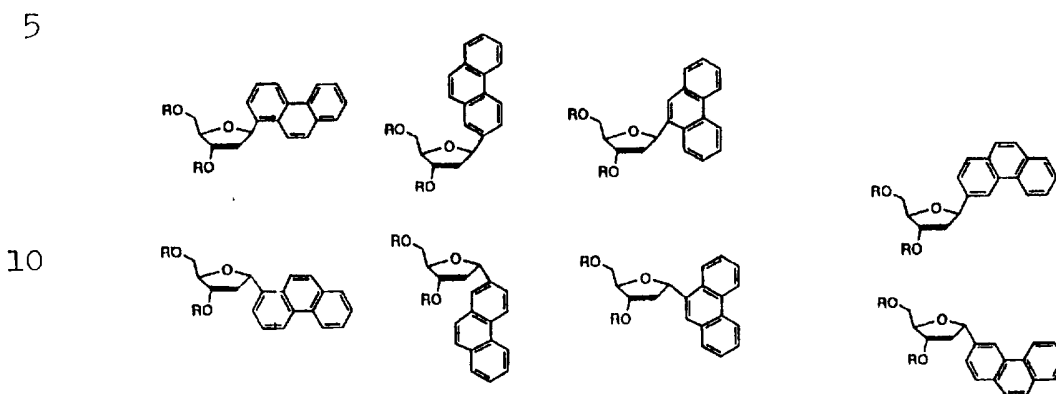
25



30

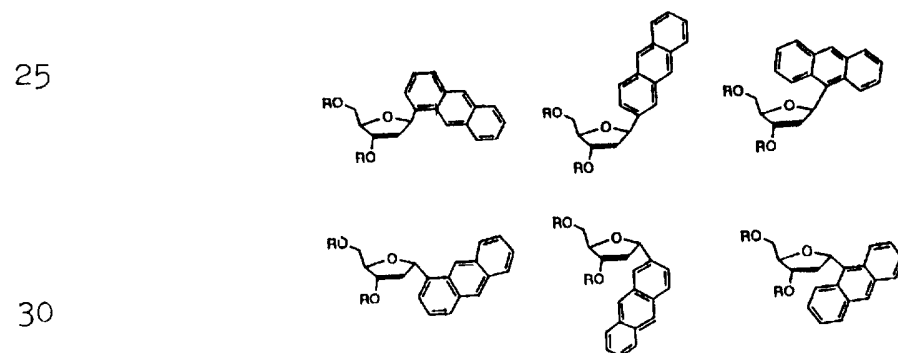
35

1 In another embodiment of the invention, the phenanthrene-derivatized deoxynucleoside has at least one of the following structures:



15

20 In yet another embodiment of the invention, the anthracene-derivatized deoxynucleoside has at least one of the following structures:

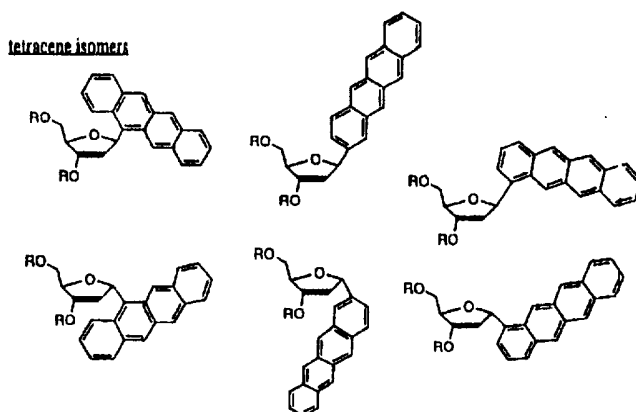


35

1

In another embodiment of the invention, the tetracene-derivatives deoxynucleoside has at least one of the following structures:

5



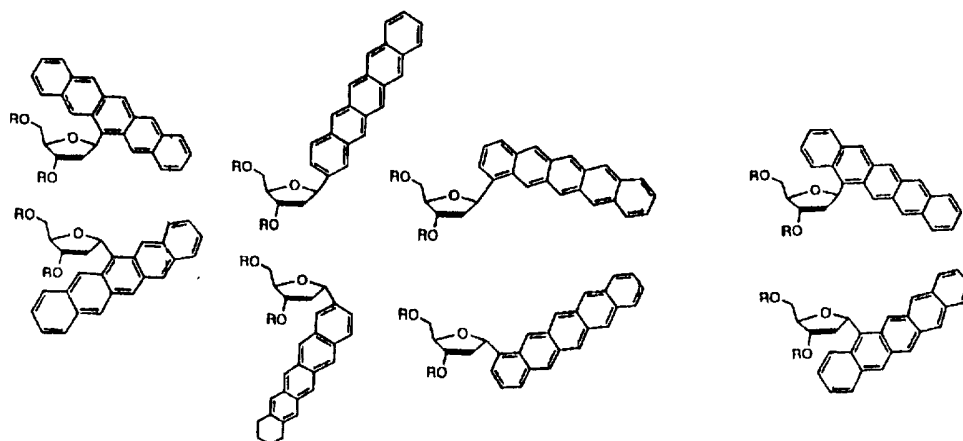
10

15

20

In still another embodiment of the invention, the pentacene-derivatized deoxynucleoside has at least one of the following structures:

25

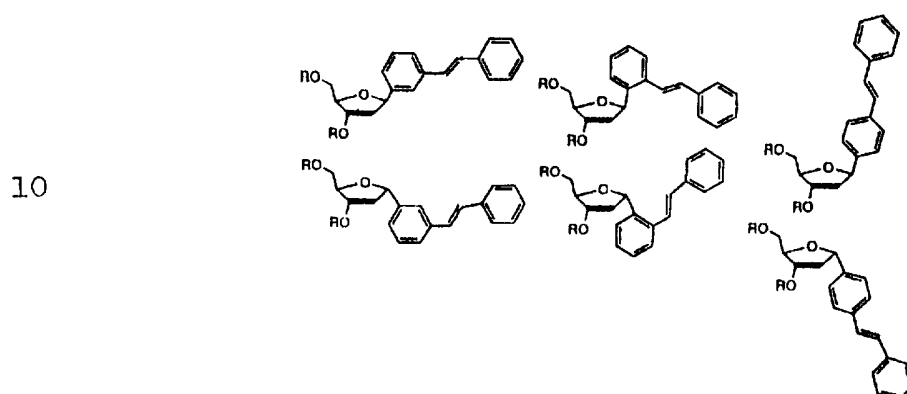


30

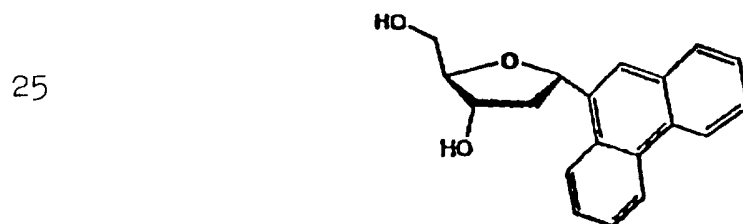
35

1 In another embodiment of the invention, the
stilbene-derivatives deoxynucleoside has at least one
of the following structures:

5

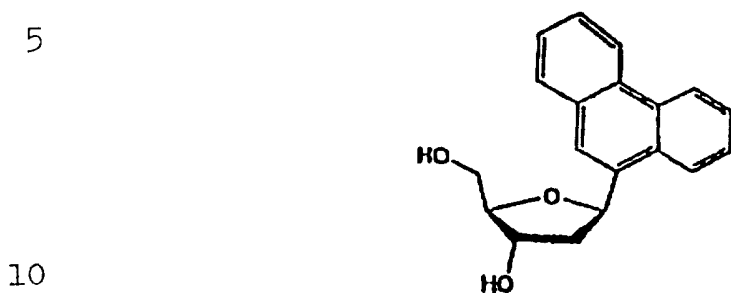


20 In yet another embodiment of the invention,
the phenanthrene-derivatized nucleoside is α -9-
phenanthrenyl deoxynucleoside having the structural
formula:

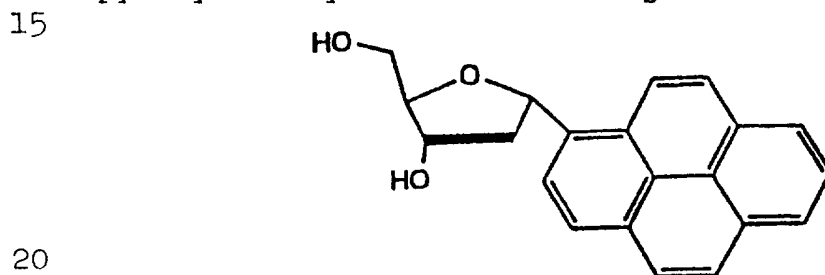


35

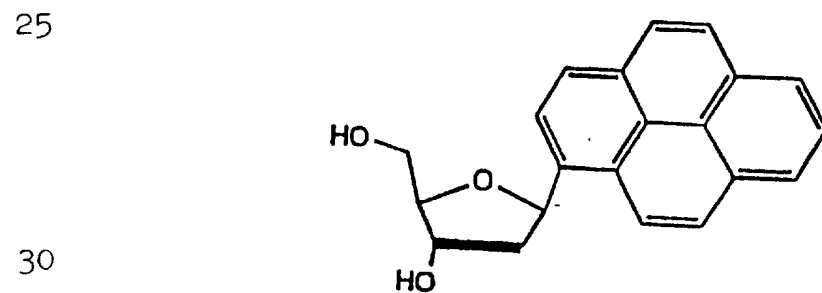
1 In another embodiment of the invention, the
phenanthrene-derivatized nucleoside is β -9-
phenanthrenyl deoxynucleoside having the structural
formula:



In still another embodiment of the invention, the
pyrene-derivatized nucleoside is α -1-
pyrenyl deoxynucleoside having the structural formula:



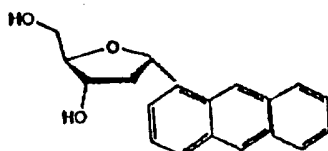
In yet another embodiment of the invention,
the pyrene-derivatized nucleoside is β -1-pyrenyl
deoxynucleoside having the structural formula:



35

1 In yet another embodiment in the invention,
the anthracene-derivatized nucleoside is α -1-
anthracenyl deoxynucleoside having the structural
formula:

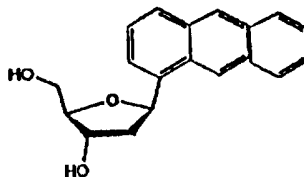
5



10

In yet another embodiment of the invention,
the anthracene-derivatized nucleoside is β -1-
anthracenyl deoxynucleoside having the structural
formula:

15



20

Useful intermediates provided by the present
invention include the adduct of Hoffer's chlorosugar
and the polycyclic aromatic hydrocarbon such as, for
example, the anthracene, phenanthrene, pyrene,
25 stilbene, tetracene, or pentacene-derivatized
nucleoside 5'-3'-paratolueyl diester.

Other particularly useful intermediates
provided by the present invention include, for
example, the pyrene, anthracene, phenanthrene,

30

35

1 stilbene, tetracene or pentacene 5'-dimethoxy trityl
ether-3'-O-H-phosphate.

5 Useful phosphoramidite derivatives provided
by the present invention include N,N-diisopropyl-O-
cyanoethyl phosphoramidite derivitized at the 3'
alcohol of the pyrene, anthracene, phenanthrene,
stilbene, tetracene or pentacene derivitized
nucleoside.

10 The subject anthracene, phenanthrene
pyrene, stilbene, tetracene or pentacene-derivitized
nucleosides, when incorporated into a nucleic acid
such as RNA or DNA, provide fluorescence at a range of
450-550 nm with a peak at 483 nm. In accordance with
the present invention, it has been surprisingly found
15 that two or more adjacent derivitized nucleosides form
bright, long-wavelength excimers while stabilizing
nucleic acid helices by their strong base stacking
properties. Thus, the multilabel quenching problem
associated with other fluorescent nucleoside tags is
avoided by the present invention while providing a
20 high sensitivity of detection.

The subject fluorescent nucleosides of the
present invention can be synthesized by coupling
polycyclic aromatic hydrocarbons to a sugar using a
modification of the organocadmium strategy described
25 in Schweitzer and Kool (1995) J. Am. Chem. Soc.
117:1863. The disclosure of this article and of all
other articles cited in this application are
incorporated herein as if fully set forth.

30 The C-nucleoside coupling involves the
reaction of organocadmium or organozinc derivatives of

35

1 the aromatic species with the well known α -chlorosugar
synthon of Hoffer¹⁹. The steps involved in the
glycosidic coupling of anthracene, pyrene,
phenanthrene, stilbene, tetracene, or pentacene to a
5 sugar is set forth in Figure 6 where Ar is an aromatic
hydrocarbon selected from the group consisting of
anthracene, phenanthrene, pyrene, stilbene, tetracene
or pentacene. This coupling results in a mixture of
alpha and beta anomers in isolated yields of between
10 about 54-81%. Alpha-anomeric C-nucleosides are the
primary reaction products.

Toluoyl protecting groups may be removed in
methanolic base. Thus, in accordance with the present
invention, free unprotected nucleosides can be
15 produced in as little as two steps: aromatic coupling
and ester deprotection.

The alpha-anomers may be converted to the
beta configuration by a third step, acid-catalyzed
equilibration. A preferred acid catalyzed
20 equilibration reaction uses benzenesulfonic acid in
refluxing xylene, in the presence of a small amount of
water. The alpha-anomers of the subject nucleoside
analogs provide greater fluorescence and may therefore
be preferred for fluorescent labeling purposes. The
present invention also provides use of a string of the
25 subject nucleoside analogs which can be attached to
generally any compound via a chemical bridge such as a
thiol group. Methods for joining molecules can be
found, for example, in S.L. Beavcage and R.P. Iyer
(1993) Tetrahedron 49:1925-1963.

30

35

1 The anthracene, phenanthrene, pyrene,
2 stilbene, tetracene or pentacene-derivatized
3 nucleosides of the present invention may be
4 incorporated into an RNA or DNA strand during
5 synthesis by any of a myriad of procedures known for
6 making DNA or RNA. For example, such procedures
7 include enzymatic synthesis and chemical synthesis.
8 Chemical synthesis include solution or solid phase
9 techniques.

10 Enzymatic methods of RNA oligonucleotide
11 synthesis frequently employ Klenow, T7, T4, Tag or E.
12 coli DNA polymerases as described in Sambrook et al.
13 (1989, Molecular Cloning: A Laboratory Manual, Cold
14 Spring Harbor Press, NY). Enzymatic methods of RNA
15 oligonucleotide synthesis frequently employ SP6, T3,
16 or T7 RNA polymerase as described in Sambrook et al.
17 Reverse transcriptase can also be used to synthesize
18 DNA from RNA (Sambrook et al.). To prepare
19 oligonucleotides enzymatically requires a template
20 nucleic acid which can either be synthesized
21 chemically, or be obtained as mRNA, genomic DNA,
22 cloned genomic DNA, cloned cDNA or other recombinant
23 DNA. Some enzymatic methods of DNA oligonucleotide
24 synthesis can require an additional primer
25 oligonucleotide which can be synthesized chemically.
26 Finally, linear oligonucleotides can be prepared by
27 PCR techniques as described, for example, by Saiki et
28 al., 1988, Science 239:487.

29 Chemical synthesis of linear
30 oligonucleotides is well known in the art and can be
31 achieve by solution or solid phase techniques.

1 Moreover, linear oligonucleotides of defined sequence
can be purchased commercially or can be made by any of
several different synthetic procedures including the
phosphoramidite, phosphite triester, H-phosphonate and
5 phosphotriester methods, typically by automated
synthesis methods. The synthesis method selected can
depend on the length of the desired oligonucleotide
and such choice is within the skill of the ordinary
artisan. For example, the phosphoramidite and
10 phosphite triester method produce oligonucleotides
having 175 or more nucleotides while the H-phosphonate
method works well for oligonucleotides of less than
100 nucleotides. If modified bases in addition to the
nucleoside base analogs of the present invention are
15 incorporated into the oligonucleotide, and
particularly if modified phosphodiester linkages are
used, then the synthetic procedures are altered as
needed according to known procedures. In this regard,
Uhlmann et al. (1990, Chemical Reviews 90:543-584)
20 provide references and outline procedures for making
oligonucleotides with modified based and modified
phosphodiester linkages.

Synthetic, linear oligonucleotides may be
purified by polyacrylamide gel electrophoresis or by
any of a number of chromatographic methods, including
25 gel chromatography and high pressure liquid
chromatography. To confirm a nucleotide sequence,
oligonucleotides may be subjected to DNA sequencing by
any of the known procedures, including Maxam and
Gilbert sequencing, Sanger sequencing, capillary
30 electrophoresis sequencing, the wandering spot

1 sequencing procedure or by using selective chemical
degradation of oligonucleotides bound to Hybond paper.
Sequences of short oligonucleotides can also be
analyzed by laser desorption mass spectroscopy or by
fast atom bombardment (McNeal, et al., 1982, J. Am.
5 Chem. Soc. 104:976; Viari, et al., 1987, Biomed.
Environ. Mass Spectrom. 14:83; Grotjahn, et al., 1982,
Nuc. Acid. Res. 10:4671). Sequencing methods are also
available for RNA oligonucleotides.

10 In a preferred method, DNA oligonucleotides
are synthesized by automated methods using a DNA
synthesizer and β -cyanoethylphosphoramidite chemistry.
Extended coupling times (10 minute) are preferably
used for anthracene, phenanthrene, pyrene, stilbene,
15 tetracene or pentacene-derivatized nucleoside
residues. Oligomers may be purified by prepative
denaturing polyacrylamide gel electrophoresis and
isolated by methods known in the art such as the crush
and soak method.

20 The subject fluorescent nucleosides of the
present invention can be incorporated into a nucleic
acid in order to achieve fluorescence labeling.
Standard methods may be used to convert the
unprotected nucleosides to 5'-dimethoxytrityl-
protected derivatives. For example, the unprotected
25 subject nucleosides may be co-evaporated with dry
pyridine, then dissolved in pyridine and
methylenechloride. A catalytic amount of DMAP, and
both diisopropylethylamine and 4,4'-dimethoxytriryl
(DMT) chloride is then added and the mixture stirred
30 at room temperature for about eight hours. Hexanes

1 are added and the mixture loaded on a flash silica gel
column and the product, 5'-dimethoxytrityl-protected
derivatives, eluted. These derivatives may then be
converted into cyanoethyl phosphoramidite derivatives
5 for incorporation into a nucleic acid sequence such as
DNA or RNA.

The preparation of 3'-O-phosphoramidites
from the 5'-dimethoxytrityl-protected anthracene,
phenanthrene, pyrene, stilbene, tetracene or
10 pentacene-derivatized nucleosides is achieved by
methods well known in the art such as, for example,
dissolving the protected nucleoside derivatives in dry
methylene chloride and adding diisopropylethylamine
and 2-cyanoethyl N,N,-
15 diisopropylchlorophosphoramidite. The reaction
mixture is stirred at room temperature for a period of
about 4 hours after which hexanes are added. The
mixture is then loaded to a flash silica gel column
and the product eluted as an oil.

20 In accordance with the present invention,
one or more of the subject nucleosides may be
incorporated at various positions in an RNA or DNA
sequence. For example, one or more subject
nucleosides may be incorporated within a stretch of
sequence so that the DNA or RNA fragment is
25 effectively tagged towards the middle of the molecule.
One or more subject nucleosides may also be
incorporated near or at the end of an RNA or DNA
sequence.

30 In another aspect of the invention,
oligonucleotides may be designed to form triplexes

1 with a single stranded target nucleic acid by folding
into a hairpin configuration so that a loop of at
least about five nucleotides separates the two
strands. One or more anthracene, phenanthrene,
pyrene, stilbene, tetracene or pentacene-derivatized
5 nucleosides may be incorporated within the loop. This
configuration is illustrated in Figure 13.

In another aspect of the invention one or
more anthracene, phenanthrene pyrene, stilbene,
tetracene, or pentacene-derivatized nucleosides may be
10 incorporated within a linear nucleic acid molecule, or
at either or both the 5' or 3' ends of a linear
nucleic acid molecule.

In a preferred embodiment, the subject
fluorescent nucleosides are present in more than one
15 position in an RNA or DNA. In a more preferred
embodiment, at least two subject nucleosides are
placed adjacent to one another within an RNA or DNA
sequence. In another preferred embodiment, at least
two subject nucleosides are placed adjacent to one
20 another within a loop of a hairpin oligonucleotide.

In another preferred embodiment, the subject
nucleosides are incorporated into a circular
oligonucleotide. The loop domains which connect two
opposing strands and thus enclose a circle have the
25 subject nucleosides incorporated within.

In a more preferred embodiment, at least one
subject nucleoside is incorporated into a DNA molecule
(hairpin, circle or linear) adjacent to an adenine (A)
base. In accordance with the present invention,
30 greater fluorescence intensities may be achieved using

1 three to five pyrenes adjacent to an A. More than
2 five total subject nucleosides may be used within a
3 nucleic acid molecule if groups of less than five of
4 such nucleosides are separated such as in three to
5 five nucleosides separated by an A, followed by three
6 to five more subject nucleosides separated by another
7 A, followed by three to five more subject nucleosides
8 (P3AP3AP3; P4AP4AP4; or P5AP5AP5). Such groups may
9 also be added to one or both ends of a nucleic acid
10 molecule.

11 In another preferred embodiment, an odd
12 number of subject fluorescent nucleosides is attached
13 to or incorporated into a nucleic acid molecule.
14 Thus, labeling a nucleic acid molecule with three,
15 five or seven subject nucleosides is especially
16 preferred.

17 The DNA and RNA sequences comprising
18 anthracene, phenanthrene, pyrene, stilbene, tetracene
19 or pentacene-derivatized nucleosides of the present
20 invention are useful for detecting target nucleic
21 acids in tissues, genomic material such as chromatin
22 and chromosomes, solutions or immobilized on
23 membranes. The fluorescently labeled nucleoside
24 derivatives of the present invention are particularly
25 useful when attached to a solid support such as
26 controlled pure glass (cpg). Thus, the DNA and RNA
27 sequences containing anthracene, phenanthrene pyrene,
28 stilbene, tetracene or pentacene-derivatized
29 nucleosides of the present invention hybridize to a
30 target nucleic acid of sufficient complementarity in
31 the detection of such targets by contacting the

1 derivitized probe with the target in a sample to be
tested for a time and under conditions sufficient to
detectably hybridize the probe with the target. The
present nucleoside derivatives are particularly useful
5 in any technique which uses fluorescent-tagged
oligonucleotides for detection. A rapidly growing
diagnostic technique which involves fluorescence
detection is fluorescence in situ hybridization
(FISH). The method uses long, enzymatically
10 synthesized DNA strands tagged with multiple
fluorescent labels. These are hybridized to fixed
chromosomes from a patient's cell, and if the gene in
question is present, a colored fluorescent spot is
visible on the chromosome by fluorescence microscopy.
15 The method is used to detect whole genes such as the
bcr/abl translocated gene in CML, or extra copies of
genes in certain genetic diseases.

Small synthetic oligonucleotides are
expected to have much higher sequence specificity than
long traditional FISH probes. For FISH to work, a
20 probe must be fluorescent-labeled brightly enough to
detect under the microscope. Thus, an oligonucleotide
must carry the equivalent of several (roughly ~ 8-40)
fluorescent tags. The fluorescent nucleosides of the
present invention give greater brightness with
25 multiple labels. This method takes advantage of
excimer emission from multiple pyrenes, for example,
stacked together.

Specific applications of the subject
30 fluorescent nucleosides in labeling nucleic acids

35

1 include: fluorescent primers for automated DNA
sequencing, fluorescent probes for flow cytometry,
fluorescent probes for ELISA-like sandwich assays,
fluorophores for measurement of protein-DNA binding,
5 fluorescent primers for detection/identification after
PCR, fluorescent probes for *in situ*
hybridization/microscopy (RNA and DNA targets),
measurement of cellular uptake of DNA, measurement of
distance, orientation and dynamics in nucleic acid
10 structures, and fluorescent probes for
Southern/Northern blots and related assays.

Complementarity between nucleic acids is the
degree to which the bases in one nucleic acid strand
can hydrogen bond, or base pair, with the bases in a
second nucleic acid strand. Hence, complementarity
15 can sometimes be conveniently described by the
percentage, i.e., proportion, of nucleotides which
form base pairs between two strands. As used herein,
"sufficient complementarity" means that a sufficient
number of base pairs exist between a target nucleic
20 acid and the anthracene, pyrene, phenanthrene,
stilbene, tetracene or pentacene labeled nucleic acid
of the present invention so that detectable binding is
achieved.

When expressed or measured by percentage of
25 base pairs formed, the degree of complementarity can
range from as little as about 30-40% complementarity
to full, i.e. 100%, complementarity. In general, the
overall degree of complementarity between the target
and labeled subject nucleic acid is preferably at
30 least about 50%.

35

1 The degree of complementarity that provides
detectable binding between the subject labeled nucleic
acids and a target is dependent upon the conditions
under which binding occurs. It is well known that
binding, i.e., hybridization, between nucleic acid
5 strands depends on factors besides the degree of
mismatch between the two sequences. Such factors
include the GC content of the region, temperature,
ionic strength, the presence of formamide, and types
of counter ions present. The effect that these
10 conditions have upon binding is known to one skilled
in the art. Furthermore, conditions are frequently
determined by the circumstance of use. Binding
conditions can be manipulated in vitro to optimize the
utility of the subject fluorescent nucleic acids. A
15 thorough treatment of the qualitative and quantitative
considerations involved in establishing binding
conditions that allow one skilled in art to design
appropriate oligonucleotides for use under the desired
conditions is provided by Belz et al., 1983 Methods
20 Enzymol. 100:266-285 and by Sambrook et al.

 Thus, for the present invention, one of
ordinary skill in the art can readily design a
fluorescently labeled nucleic acid sequence having
one or more fluorescent nucleosides selected from the
25 group consisting of anthracene, phenanthrene, pyrene,
stilbene, tetracene and pentacene-derivatized
nucleosides which exhibit sufficient complementarity
to detectably bind to its target sequence. As used
herein, "binding" or "stable binding" means that a
30 sufficient amount of the nucleic acid is bound to its

1 target to permit detection of that binding. In
accordance with the present invention, binding can be
detected by either physical or functional properties
of the subject nucleic acid : target complex.

5 Binding between a target and a nucleic acid
can be detected by any procedure known to one skilled
in the art, including both functional or physical
binding assays. Binding may be detected functionally
by determining whether binding has an observable
effect upon a biosynthetic process such as DNA
10 replication, RNA transcription, protein translation or
the like.

Physical methods of detecting the binding of
complementary strands of DNA or RNA are well known in
the art, and include such methods as DNase I or
15 chemical footprinting, gel shift and affinity clavage
assays, Northern blotting, dot blotting and light
absorption detection procedures. Fluorescence
methodologies include spectroscopy, fluorescence
spectrophotometry and fluorescence assisted cell
20 sorting (FACS), fluorescence microscopy, and digital
imaging camera.

The binding between an oligonucleotide and
its target nucleic acid is frequently characterized by
the temperature at which 50% of the oligonucleotide is
25 melted from its target. This temperature is the
melting temperature (T_m). The higher T_m means a
stronger or more stable complex relative to a complex
with a lower T_m .

In accordance with the present invention,
30 observing fluorescence in the 300-900 nm range with

1 the appropriate hybridization and washing conditions
indicate binding of a fluoroscently labeled nucleic
acid of the present invention to its target sequence.

5 In accordance with the present invention,
binding of anthracene, pyrene, phenanthrene, stilbene,
tetracene or pentacene-labeled nucleic acids to a
target sequence can be observed by native gel shift
experiments where the bound complexes are clearly
visible under fluorescent light. For example, a
10 nucleic acid: target complex having a single pyrene-
based nucleoside appears deep blue in color while a
nucleic acid: target complex having multiple pyrene-
based nucleosides appear light blue to white in
appearance, indicative of longer wavelength emission.

15 The following examples further illustrate
the invention. For purposes of the following examples,
compounds 1-6 and compounds 1a-6a are provided in Fig.
5. Compounds 1-6 are the β form of the molecule as
shown and compounds 1a-6a are the α form of the
molecule (not shown) in the Figure. All reference to
20 compounds 1-6 and 1a-6a in the text therefore refer to
those depicted structurally in Fig. 5.

25

30

35

EXAMPLE 1

SYNTHESIS

1

The previously described method of C-nucleoside coupling¹³ was utilized to generate the new aromatic nucleosides 1-4 (Fig. 5) as their bis-toluoyl esters (Fig. 6). The method involves the reaction of organocadmium derivatives of the aromatic species with the well-known α -chlorosugar synthon of Hoffer¹⁹ (Fig. 6).

10

Glycosidic coupling reaction and isolation of major α -epimers as bis-p-toluoyl esters of 1a-6a.

Dry THF (5 mL) was placed in a round-bottomed flask equipped with a condenser, drying tube and addition funnel. Magnesium turnings (0.3 g, 1.2 mmol) and a few crystals of iodine were added. 1-Bromopyrene (0.35g, 1.2 mmol) was added to the mixture. Slight heating was needed (40°C) to drive the reaction to completion. After formation of the Grignard reagent was complete (~1 hr), dry CdCl₂ (110mg, 0.6 mmol) was added and the reaction mixture was continuously heated under reflux for 1 hr. 1'- α -chloro-3',5'-di-O-toluoyl-2'-deoxyribose¹⁹ (0.51 g, 1.3 mmol) was then added to the above mixture in one portion. The solution was stirred at room temperature for 4 hr under an atmosphere of N₂. The solution was poured into 10% ammonium chloride (2 x 50 mL) and extracted with methylene chloride. The organic layers were washed with saturated sodium bicarbonate and brine and dried over anhydrous magnesium sulfate. The

15

20

25

30

35

1 solution was filtered, concentrated and purified by
flash silica gel chromatography eluting with hexanes-
ethyl acetate (9:1). The major product 1a bis-toluoyl
ester was obtained as a pale yellow oil (α -epimer, 48%
isolated yield): $^1\text{H NMR}$ (CDCl_3 , ppm) δ 8.80 (2H, d,
5 $J=8.0$), 8.72 (2H, d, $J=8.0$) 8.05 (1H, s), 7.92-8.00
(2H, m), 7.72-7.60 (4H, m), 7.58 (2H, d, $J=8.0$), 7.32
(2H, d, $J=8.0$), 6.96 (2, d, $J=8.0$), 6.16 (1h, dd, $J=$
8.2, 6.0), 5.76 (1H, m), 4.98 (1H, m), 4.75-4.65 (2H,
m), 3.30-3.22 (1H, m), 3.50-3.45 (1H, m), 3.44 (3H,
10 s), 3.38 (3H, s); $^{13}\text{C NMR}$ (CDCl_3 , ppm) δ 21.3, 21.4,
39.2, 64.5, 76.2, 78.0, 82.5, 122.3, 122.9, 123.2,
123.6, 126.0, 126.3, 126.4, 126.6, 126.8, 127.0,
128.7, 128.8, 128.9, 129.0, 129.2, 129.4, 129.6,
129.8, 130.6, 131.4, 136.2, 143.5, 143.6, 165.8,
15 166.2; HRMS (FAB, 3-NBA matrix) calculated for
 $\text{C}_{37}\text{H}_{31}\text{O}_5$ (M+1) 554.2093, found 554.2069.

2a bis-toluoyl ester (α -epimer, 43% isolated
yield): $^1\text{H NMR}$ (CDCl_3 , ppm) δ 8.80 (2H, d, $J=8.0$), 8.72
(2H, d, $J=8.0$), 8.05 (1H, s), 7.92-8.00 (2H, m), 7.72-
20 7.60 (4H, m), 7.58 (2H, d, $J=8.0$), 7.32 (2H, d,
 $J=8.0$), 6.96 (2H, d, $J=8.0$), 6.15 (1H, dd, $J=8.2$,
6.0), 5.76 (1H, m), 4.98 (1H, m), 4.75-4.65 (2H, m),
3.30-3.22 (1H, m), 3.50-3.45 (1H, m), 3.44 (3H, s),
3.38 (3H, s); $^{13}\text{C NMR}$, (CDCl_3 , ppm) δ 21.3, 21.4, 39.2,
25 64.5, 76.2, 78.0, 82.5, 122.3, 122.9, 123.2, 123.6,
126.0, 126-3, 126.4, 126.6, 126.8, 127.0, 128.7,
128.8, 128.9, 129.0, 129.2, 129.4, 129.6, 129.8,
130.6, 131.4, 136.2, 143.5, 143.6, 165.8, 166.2; HRMS
(FAB, 3-NBA matrix) calculated for $\text{C}_{35}\text{H}_{31}\text{O}_5$ (M+1)
30 531.2172, found 531.2174.

3a bis-toluoyl ester (α -epimer, 52% isolated
1 yield): ^1H NMR (CDCl_3 , ppm) δ 8.05 (2H, d, $J=8.0$), 7.95
(2H, m), 7.83 (2H, overlapped d), 7.71 (2H, d, $J=8.0$),
7.55 (3H, m), 7.32 (2H, d, $J=8.0$), 7.19 (2H, d, $J=$
5 8.0), 6.10 (1H, dd, $J=8.0, 6.0$), 5.69 (1H, m), 4.90
(1H, m), 4.76-4.65 (2H, m), 3.28-3.18 (1H, m), 2.52-
2.45 (1H, m), 2.48 (3H, s), 2.42 (3H, s); ^{13}C NMR
(CDCl_3 , ppm) δ 21.4, 21.5, 39.5, 64.5, 76.2, 77.8,
82.2, 122.1, 122.9, 125.1, 125.3, 125.8, 126.6, 127.0,
128.7, 128.8, 128.9, 129.2, 129.4, 129.5, 129.9,
10 133.6, 137.9, 143.6, 165.8, 166.2; HRMS ((FAB, 3-NBA
matrix) calcd for $\text{C}_{31}\text{H}_{29}\text{O}_5$ ($M+1$) 481.2015, found
481.2025.

4a bis-toluoyl ester (α -epimer, 31% isolated
15 yield): ^1H NMR (CDCl_3 , ppm) δ 8.02 (2H, d, $J=8.0$), 7.92-
7.97 (4H m), 7.83 (2H, d, $J=8.0$), 7.52-7.60 (3H, m),
7.32 (2H, d, $J=8.0$), 7.05 (2H, d, $J=8.0$), 5.72 (1H,
m), 5.62 (1H, dd, $J=8.2, 6.0$), 4.85 (1H, m), 4.76-4.65
(2H, m), 3.12-3.02 (1H, m), 2.52-2.45 (1H, m), 2.42
(3H, s), 2.38 (3H, s); ^{13}C NMR (CDCl_3 , ppm) δ 21.3,
20 21.4, 40.0, 64.4, 76.3, 80.2, 82.1, 122.1, 122.9,
125.1, 125.3, 125.8, 126.6, 127.0, 128.7, 128.8,
128.9, 129.2, 129.4, 129.5, 129.9, 133.6, 139.9,
143.8, 165.8, 166.3; HRMS (FAB, 3-NBA matrix)
calculated for $\text{C}_{31}\text{H}_{29}\text{O}_5$ ($M+1$) 481.2043, found 481.2015.

5a bis-toluoyl ester (α -epimer, 13% isolated
25 yield): ^1H NMR (CDCl_3 , ppm) δ 8.02 (2H, d, $J=8.0$), 7.86
(1H, d, $J=8.0$), 7.45 (1H, s), 7.23-7.28 (5H, m), 6.95
(1H, s), 5.69 (1H, br s), 5.54 (1H, dd, $J=8.0, 6.0$),
4.81 (1H, br s), 4.69-4.56 (2H, m), 3.07-2.98 (1H, m),
30 2.43 (6H, s), 1.35 (3H, s); ^{13}C NMR (CDCl_3 , ppm) δ

18.3, 19.0, 19.1, 21.4, 39.1, 64.4, 76.3, 77.2, 81.6,
125.9, 126.7, 126.8, 128.8, 128.9, 129.5, 131.1,
131.5, 133.7, 135.0, 137.3, 143.5, 143.6, 165.9, 166.1;
HRMS (FAB, 3-NBA matrix) calculated for $C_{30}H_{33}O_5$ (M+1)
472.2250, found 472.2234.

5 6a bis-toluoyl ester (α -epimer, 16% isolated
yield): 1H NMR ($CDCl_3$, ppm) δ 8.0 (2H, d, $J=8.0$), 7.72
(2H, d, $J=8.0$), 7.43 (1H, t, $J=8.5$), 7.27 (2H, d,
 $J=8.0$), 7.19 (2H, d, $J=8.0$), 6.76 (1H, d, $J=8.0$), 5.61
10 (1H, br s), 5.57 (1H, dd, $J=8.0, 6.0$), 4.74 (1H, br
s), 4.57, (2H, t $J=5.0$), 3.02-2.93 (1H, m), 2.43 (3H,
s), 2.23 (3H, s); ^{13}C NMR ($CDCl_3$, ppm) δ 14.0, 20, 21.6,
39.4, 64.5, 74.8, 76.3, 82.5, 103.1 (t) 120.1 (dd),
125.2 (dd), 126.5, 126.8, 128.8, 129.9, 129.3, 129.5,
143.6, 143.8, 158.6, 158.7; HRMS (FAB, 3-NBA matrix)
15 calculated for $C_{28}H_{26}F_2O_5Na$ 503.1646, found 503.1636.

Epimerization of 1',2'-dideoxy-1'- α -aryl-3',5'-
di-0-toluoyl-D-ribofuranoses and isolation of β -
epimers.

20 A solution of 6a-bis-toluoyl ester (780 mg,
1.62 mmol) in toluene (50 mL) was added catalytic
amount of benzenesulfonic acid (-10%), 1 drop of
concentrated H_2SO_4 and 2-4 drops of H_2O . The reaction
mixture was refluxed under vigorous stirring for 4-6
25 hrs. The mixture was then poured into 5% aqueous
 $NaHCO_3$ (50 mL) and extracted with EtOAc (3 x 50 mL).
The combined organic layers were dried over anhydrous
 $MgSO_4$ and evaporated. Flash column chromatography
(eluent solution: 8:1 to 2:1 hexanes:EtOAc) of the
30 crude mixmm gave 430 mg of 6-bis-toluoyl ester (β -

epimer, 46% isolated yield): ^1H NMR (CDCl_3 , ppm) δ 8.0
1 (4H, 2 x d, $J=8.0$), 7.35-7.25 (5H, m), 6.76 (1H, t,
 $J=10.0$), 5.64 (1H, d, $J=5.2$), 5.46, (1H, dd, $J=10.2$,
2.3), 4.78 (1H, dd, $J=1.9$, 11.8), 4.63 (1H, dd,
5 $J=1.9$, 11.8), 4.54 (1H, m), 2.64 (1H, dd, $J=2.6$,
11.8), 2.43 (3H, s), 2.46 (3H, s), 2.23 (1H, m); ^{13}C
NMR (CDCl_3 , ppm), δ 13.8, 22.0 (d), 40.1, 64.9, 74.9,
83.0, 103.0 (t), 120.1 (d), 124.5 (d), 127.3 (d),
128.6, 128.8 (d), 128.9 (d), 144.0 (d), 156.5 (d),
158.0 (d), 155.9 (d), 162.3 (d), 166.1 (d); HRMS (FAB,
10 3-NBA matrix) calculated for $\text{C}_{28}\text{H}_{26}\text{F}_2\text{O}_5$ 481.1827, found
481.1853.

1 bis-toluoyl ester (β -epimer, 38% isolated
yield): ^1H NMR (CDCl_3 , ppm) δ 8.36 (1H, d, $J=7.9$), 8.31
15 (1H, d, $J=7.9$), 8.20-8.17 (3H, m), 8.13-8.05 (6H, m),
8.02 (2H, d, $J=8.0$), 7.37 (2H, d, $J=8.0$), 72.6 (2H,
d, $J=8.0$), 6.34 (1H, dd, $J=3.6$, 10.8), 5.78 (1H, d,
 $J=5.4$), 4.84-4.88 (2H, m), 4.78-4.76 (1H, m), 2.94
(1H, dd, $J=2.5$, 13.9), 2.50 (3H, s), 2.46 (1H, m),
2.40 (3H, s); ^{13}C NMR (CDCl_3 , ppm), δ 21.4, 21.5, 41.3,
20 64.5, 77.1, 77.9, 82.7, 122.0, 122.4, 124.5, 124.8,
125.0, 125.6, 126.8, 126.9, 127.0, 127.2, 127.3,
127.5, 128.9, 129.0, 129.5, 129.6, 130.3, 130.6,
131.1, 133.9, 143.5, 143.9, 166.0, 166.2; HRMS (FAB,
3-NBA matrix) calculated for $\text{C}_{37}\text{H}_{31}\text{O}_5$ (M+1) 554.2093,
25 found 554.2069.

2 bis-toluoyl ester (β -epimer, 28% isolated
yield): ^1H NMR (CDCl_3 , ppm) δ 8.78 (1H, d, $J=7.9$), 8.70
(1H, d, $J=7.9$), 8.13-8.09 (4H, m), 8.03 (2H, d,
 $J=8.0$), 7.84 (1H, d, $J=7.9$), 7.77-7.60 (4H, m), 7.36
30 (2H, d, $J=8.0$), 7.18 (2H, d, $J=8.0$), 6.60 (1H, dd,

1 J=3.5,10-5), 4.90 (1H, dd, J=1.9, 11.8), 4.84 (1H, dd,
J=1.9, 11.8), 2.94 (1H, dd, J=5.1, 13.7), 2.49 (3H,
s), 2.44-2.41 (1H, m), 2.38 (3H, s); ¹³C NMR (CDC13,
ppm) δ 21.4, 21.5, 40.4, 64.3, 76.9, 77.7, 82.4,
122.1, 122.8, 123.1, 123.4, 126.1, 126.4, 126.5,
5 126.8, 126.9, 128.7, 128.9, 129.0, 129.4, 129.5,
129.6, 129.8, 130.4, 131.3, 134.7, 143.5, 143.9,
166.0, 166.2; HRMS (FAB, 3-NBA matrix) calculated for
C₃₅H₃₁O₅ (M+1) 531.2172, found 531.2174.

10 **3 bis-toluoyl ester** (β-epimer, 37% isolated
yield): ¹H NMR (CDCl₃, ppm) δ 8.09-8.04 (3H, m), 7.97
(1H, d, J=8.0), 7.91 (1H, overlapped d, J=6.3, 6.2),
7.88 (1H, d, J=8.0), 7.51 (2H, overlapped d, J=6.7,
6.5), 7.46 (1H, d, J=7.9), 7.34 (2H, d, J=8.0), 7.22
(2H, d, J=8.0), 6.02 (1H, dd, J=3.2, 10.7), 5.71 (1H,
15 d, J=5.9), 4.78-4.78 (2H, m), 4.70-4.71 (1H, m),
2.85 (1H, dd, J=2.5, 13.8), 2.48 (3H, s), 2.47 (3H, s), 2.39-
2.37 (1H, m); ¹³C NMR (CDCl₃, ppm) δ 21.4, 21.5, 40.6,
64.5, 77.0, 77.7, 82.4, 122.1, 122.8, 125.3, 125.4,
125.7, 125.9, 126.8, 126.9, 127.9, 128.6, 128.9,
20 129.0, 129.5, 130.2, 133.4, 136.3, 143.5, 143.9,
166.0, 166.2; HRMS (FAB, 3-NBA matrix) calculated for
C₃₁H₂₉O₅ (M+I) 481.2015, found 481.2025.

25 **4 bis-toluoyl ester** (β-epimer, 41% isolated
yield): ¹H NMR (CDCl₃, ppm) δ 8.06 (2H, d, J=8.0), 8.02
(2H, d, J=8.0), 7.91-7.77 (4H, m), 7.57-7.48 (3H, m),
7.32 (2H, d, J=8.0), 7.23 (2H, d, J=8.0), 5.70 (1H, d,
J=5.7), 5.46 (1H, dd, J=1.6, 10.9), 4.77-4.75 (2H, m),
4.66-4.65 (1H, m), 2.66 (1H, dd, J=2.6, 13.8), 2.48
(3H, s), 2.42 (3H, s); ¹³C NMR, (CDCl₃, ppm) δ 21.3,
30 21.4, 41.6, 64.5, 77.1, 80.7, 82.9, 123.5, 124.5,

125.6, 125.9, 126.8, 126.9, 127.4, 127.7, 128.1,
1
128.9, 129.3, 129.4, 129.5, 132.9, 133.0, 137.9,
143.5, 143.8, 165.9, 166.2; HRMS (FAB, 3-NBA matrix)
calculated for $C_{31}H_{29}O_5$ (M+1) 481.2043, found 481.2015.

5 bis-toluoyl ester (β -epimer, 54% isolated
5 yield): 1H NMR ($CDCl_3$, ppm) δ 8.02 (4H, 2 x d, $J=8.0$),
7.35-7.23 (5H, m), 6.92 (1H, s), 5.62 (1H, d, $J=5.6$),
5.42 (1H, dd, $J=3.5, 10.8$), 4.78 (1H, dd, $J=1.9,$
11.8), 4.70 (1H, dd, $J=1.8, 11.8$), 4.55 (1H, m), 2.56
(1H, dd, $J=2.5, 14.0$), 2.43 (3H, s), 2.46 (3H, s),
10 2.23 (1H, m); ^{13}C NMR ($CDCl_3$, ppm) δ 18.2, 19.5,
22.1 (d), 41.0, 65.0, 82.5, 126.2, 127.0 (d); 128.6
(d), 128.8 (d), 132.1 (d), 135.5, 136.2, 141.0,
144.5, 165.5, 166.0; HRMS (FAB, 3-NBA matrix)
calculated for $C_{30}H_{32}O_5$ 472.2250, found 472.2234.

15

Deprotection of 1',2'-dideoxy-1'-aryl-3',5'-di-O-
toluoyl- β -D-ribofuranoses.

A solution of 1 bis-toluoyl ester (360 mg, 0.65
mmol) in methanol (5 mL) was added NaOMe (in methanol,
20 25%, 0.5 mL, 3 eq). The reaction mixture was stirred
for 4-6 hr. Solid ammonium chloride was added until
the pH was 8. The mixture was then poured into water
and extracted with EtOAc (3 x 15 mL). The combined
organic) layers were dried over anhydrous $MgSO_4$ and
25 evaporated. Flash column chromatography (eluent;
EtOAc) of the crude mixture gave 165 mg of nucleoside
1 (β -epimer, 78%); 1H NMR ($CDCl_3$, ppm) δ 8.35 (1H, d,
 $J=8.0$), 8.31-8.14 (4H, m), 8.08-8.02 (3H, m), 6.25
(1H, dd, $J=2.8, 10.4$), 4.62 (1H, m), 4.28 (1H, m),
30 4.02-3.98 (2H, m), 2.64 (1H, dd, $J=1.0, 2.6, 13.4$),

35

1 2.02 (2H, broad s, 2 x OH); ¹³C NMR (CDCl₃, ppm) δ
44.5, 63.8, 74.3, 78.2, 88.9, 123.3, 123.7, 125.5,
125.6, 125.7, 126.0, 127.9, 128.1, 128.2, 128.3,
128.4, 128.5, 128.6, 131.7, 131.8, 136.5; HRMS (FAB,
5 3-NBA matrix) calculated for C₂₃H₂₀O₃ 318.1256, found
318.1251.

nucleoside 2, (β-epimer, 74%): ¹H NMR (CDCl₃,
ppm) δ 8.78 (1H, d, J=8.0), 8.68 (1H, d, J=8.0), 8.12
(1H, d, J=8.0), 7.90 (2H, m), 7.77-7.62 (4H, m), 5.95
(1H, dd, J=2.8, 10.4), 4.59 (1H, m), 4.22 (1H, m),
10 4.0 (1H, dd, J=3.2, 13.2), 3.95 (1H, dd, J=3.1, 13.4),
2.62 (1H, ddd, J=1.0, 5.2, 13.4), 2.25 (1H, m), 1.6
(2H, broad s, 2 x OH); ¹³C NMR (CDCl₃, ppm) δ 43.5,
63.8, 74.0, 78.0, 88.5, 123.4, 123.5, 124.0, 124.8,
127.1, 127.3, 127.4, 127.5, 129.6, 130.8, 131.0,
15 131.6, 132.8, 137.1; HRMS (FAB, 3-NBA matrix) calculated
for C₁₉H₁₈O₃ 294.1256, found 294.1250.

nucleoside 3 (β-epimer, 50%): ¹H NMR (CDCl₃, ppm)
δ 8.06 (1H, d, J=8.0), 7.88 (1H, d, J=8.0), 7.80 (1H,
d, J=8.0), 7.66 (1H, d, J=8.0), 7.55-7.46 (3H, m),
20 5.92 (1H, dd, J=2.6, 10.0), 4.52 (1H, m), 4.15 (1H,
m), 3.92-3.86 (2H, m), 2.54 (1H, dd, J=2.8, 13.3),
2.18 (1H, m), 2.02 (2H, broad s, 2 x OH); ¹³C NMR
(CDCl₃, ppm) δ 43.0, 63.0, 74.0, 77.0, 123.2, 124.0,
125.2, 125.4, 127.0, 127.5, 130.2, 134.8, 138.0;
25 HRMS (FAB, 3-NBA matrix) calculated for C₁₅H₁₆O₃
244.1099, found 244.1105.

nucleoside 4 (β-epimer, 68%): ¹H NMR (CDCl₃, ppm)
δ 7.85-7.80 (4H, m), 7.50-7.42 (3H, m), 5.35 (1H, dd,
J=2.8, 10.2), 4.43 (1H, m), 4.06 (1H, m), 3.77 (2H, m),
30 2.6 (2H, broad s, 2 x OH), 2.33 (1H, ddd, J=1.0, 5.6,

13.4), 2.02 (1H, m); ^{13}C NMR (CDCl_3 , ppm) δ 44.6, 63.9,
1 74.3, 81.5, 89.1, 125.1, 125.6, 126.6, 128.4, 128.6,
128.9, 134.3. 134.5, 140.4; HRMS (FAB, 3-NBA matrix)
calculated for $\text{C}_{15}\text{H}_{16}\text{O}_3$ 244.1099, found 244.1110.

nucleoside 5 (β -epimer, 93%): ^1H NMR (CDCl_3 , ppm)
5 δ 7.20 (1H, s), 6.97 (1H, s), 5.38 (1H, dd, $J=2.8$,
10.4), 4.43 (1H, m), 4.01 (1H, m), 3.82 (2H, m), 2.32
(3H, s), 2.26 (3H, s), 2.24 (3H, s), 1.99 (1H, m),
1.90 (2H, broad s, 2 x OH); ^{13}C NMR (CDCl_3 , ppm) δ
10 19.0, 19.5, 19.6, 41.6, 63.0, 74.0, 77.0, 87.0, 126.2,
126.3, 131.2, 131.6. 134.1. 135.2, 137.0; HRMS (FAB,
3-NBA matrix) calculated for $\text{C}_{14}\text{H}_{20}\text{O}_3$ 237.1491, found
237.1484.

nucleoside 6 (β -epimer, 89%): ^1H NMR (CDCl_3 , ppm)
 δ 7.46 (1H, t, $J=10$), 6.82 (1H, t, $J=10$), 5.31 (1H,
15 dd, $J=2.8$, 10.4), 4.32 (1H, m), 3.92 (1H, m), 3.68
(2H, m), 2.22 (3H, s), 1.89 (1H, m), 1.78 (2H, broad
s, 2 x OH); ^{13}C NMR (CDCl_3 , ppm) δ 12.2, 41.9, 62.2,
72.5, 73.2, 87.2, 101.59, 101.9, 102.3, 119.8, 119.9,
120.1, 124.1, 124.3, 128.8, 128.9, 129.0; HRMS (FAB,
20 3-NBA matrix) calculated for $\text{C}_{12}\text{H}_{14}\text{F}_2\text{O}_3\text{Na}$ 267.0809,
found 267-0812.

Preparation of 5'-O-tritylated β -C-nucleosides

Synthesized nucleoside 1 (165 mg, 0.52 mmol) was
25 co-evaporated with dry pyridine (4 mL) twice and then
dissolved in 5 mL of pyridine and 4 mL of
methylenechloride. To the above mixture was added
catalytic amount of DMAP, diisopropylethylamine (0.14
mL, 1.5 eq) and 4,4'-dimethoxytrityl (DMT) chloride
30 (320 mg, 1.8 eq). The mixture was stirred at room

1 temperature for 8 h. Hexanes (5 mL) was added and the
mixture was loaded on flash silica gel column (pre-
equilibrated with 5% triethylamine in hexanes) and
eluted (5:1 Hexanes:EtOAc to 2:1 Hexanes:EtOAc). The
product 1 DMT ether was obtained as a yellowish foam
5 in 64% yield (200 mg, 0.32 mmol): ^1H NMR (CDCl_3 , ppm) δ
8.34 (2H, overlapped d, $J=8.0$), 8.24-8.02 (7H, m),
7.56 (2H, overlapped d, $J=8.0$), 7.45-7.27 (7H, m),
6.86 (4H, d, $J=8.0$), 6.52 (1H, d, $J=6.2$), 6.24 (1H,
dd, $J=2.6, 10.4$), 4.60 (1H, m), 4.30 (1H, m), 3.81
10 (6H, s), 3.56 (2H, m), 2.64 (1H, m), 2.30 (1H, m). ^{13}C
NMR 400MHz (CDCl_3 , ppm) δ 43.9, 55.2, 64.5, 74.5,
77.5, 86.4 (d), 113.2, 122.8, 123.0, 124.8 (d), 125.0,
125.2, 125.9, 126.9, 127.2, 127.5, 127.6, 127.7,
128.0, 128.4, 130.2, 130.3, 130.6 (d), 131.5, 135.5,
15 136.1, 145.0, 158.5; HRMS (FAB, 3-NBA matrix)
calculated for $\text{C}_{42}\text{H}_{36}\text{O}_5$, 620.2564, found 620.2563.

2 DMT ether (280 mg, 59%): ^1H NMR (CDCl_3 , ppm) δ
8.78 (1H, d, $J=8.0$), 8.68 (1H, d, $J=8.0$), 8.07 (2H,
m), 7.8 (1H, d, $J=8.0$), 7.80-7.24 (12H, m), 6.84 (4H,
20 overlapped d, $J=8.0$), 5.94 (1H, dd, $J=2.9, 10$), 4.52
(1H, m), 4.22 (1H, m), 3.8 (6H, s), 3.50 (2H, m), 2.61
(1H, ddd, $J=1.0, 5.2, 13.4$), 2.25 (1H, m): ^{13}C NMR
400MHz (CDCl_3 , ppm) δ 42.6, 55.1, 64.2, 74.1, 77.1
(obscured by solvent), 85.6, 86.0, 113.1, 122.3,
25 122.8, 123.2, 124.0, 126.2, 126.4, 126.5 (d), 126.8,
127.8, 128.2, 128.8, 129.7, 129.9, 130.1, 131.5, 136.0
(d), 136.2, 144.9, 155.8; HRMS (FAB, 3-NBA matrix)
calculated for $\text{C}_{40}\text{H}_{36}\text{O}_5$, 596.2564, found 596.2563.

3 DMT ether (50 mg, 52%): ^1H NMR (CDCl_3 , ppm) δ
30 8.15 (1H, d, $J=8.0$), 7.9 (1H, d, $J=8.0$), 7.8 (1H, d,

1 J=8.0), 7.65 (1H, d, J=8.0), 7.49-7.45 (3H, m), 5.94
(1H, dd, J=2.9, 10), 4.53 (1H, m), 4.25 (1H, m), 3.8
(3H, s), 3.42 (2H, m), 3.02 (3H, s), 2.58 (1H, ddd,
5 J=1.0, 5.2, 13.4), 2.18, (1H, m); ¹³C NMR 400MHz
(CDCl₃; ppm) δ 42.9, 55.1, 64.3, 74.3, 77.1 (obscured
by solvent), 85.8, 86.2, 113.1, 122.2, 123.4, 125.4,
125.5, 125.9, 126.8, 127.7, 127.8, 128.2, 128,7,
130.1, 130.4, 133.6, 136.0, 137.7, 144.9, 158.4; HRMS
(FAB, 3-NBA matrix) calculated for C₃₆H₃₄O₅ 546.2407,
found 546.2406.

10 4 DMT ether (200 mg, 66%): ¹H NMR (CDCl₃, ppm) δ
7.83-7.94 (4H, m), 7.56-7.27, (11H, m), 6.87 (4H,
overlapped d, J=8.0), 5.41 (1, dd, J=2.9, 10), 4.52
(1H, m), 3.82 (6H, s), 3.42 (2H, m), 2.38 (1H, dd,
15 J=2.7, 13.4), 2.21 (1H, m).

5 DMT ether (311 mg, 92%): ¹H NMR (CDCl₃, ppm) δ
7.52 (2H, d, J=8.0), 7.43-7.24 (7H, m), 6.94-6.84 (6H,
m), 5.34 (1H, dd, J=2.9, 9.8), 4.42 (1H, m), 4.18 (1H,
m), 3.80 (6H, s), 3.40 (2H, m), 2.64 (1H, m), 2.29
(3H, s), 2.23 (3H, s), 2.18 (3H, s), 2.0 (1H, m); ¹³C
20 NMR (CDCl₃, ppm) δ 19.6, 19.8, 19.9, 43.1, 55.0, 64.0,
75.0, 82.2, 116.2, 125.3, 125.4, 125.6, 125.7,
130.01, 132.1, 132.2, 134.2, 135.2, 136.5, 145.0,
158.2; HRMS (FAB, 3-NBA matrix) calculated for C₃₅H₃₇O₅
538.2719, found 538-2690.

25 6 DMT ether (350 mg, 88%): ¹H NMR (CDCl₃, ppm) δ
7.46 (1H, d, J=8.0), 7.39-7.24 (5H, m), 6.83 (2H,
overlapped d, J=8.0), 6.74 (1H, dd, t=9.8, 8.6), 5.38
(1H, dd, J= 2.9, 9.9), 4.42 (1H, m), 4.06 (1H, m),
3.80 (6H, s), 3.38 (2H, m), 2.38 (1H, dd, J=2.5,
30 13.4). 21 (1H, m); ¹³C NMR (CDCl₃, ppm) δ 29.4, 42.5,

1 54.9, 64.3, 73.3, 74.3, 85.65, 102.3, 102.7, 103.0,
112.8, 115.0, 126.5, 127.5, 1127.6, 127.9, 129.1,
129.8, 128.9, 135.0, 145.0. 158.9; HRMS (FAB, 3-NBA
matrix) calcd for $C_{33}H_{32}F_2O_5$ 569.2115, found 569.2131.

5 Preparation of 3'-O-phosphoramidites

The 5'-O-tritylated compound 1 DMT ether
(200mg, 0.32 mmol) was dissolved in 4mL of dry
methylene chloride and this was added
diisopropylethylamine (0.22 mL, 1.2 mmol) and 2-
10 cyanoethyl *N,N*, - diisopropylchlorophosphoramidite
(0.11 mL, 0.48 mmol). The reaction mixture was
stirred at room temperature for 4h Hexanes (4 mL) was
added and the mixture was loaded to the flash silica
gel column (pre-equilibrated with 5% triethylamin in
15 hexanes) and eluted. The product was obtained as an
oil DMT phosphoramidite 1 (210 mg, 81 %). 1H NMR
($CDCl_3$, ppm) δ 8.44-8.33 (2H, m), 8.25-8.00 (7H, m),
7.62-7.22 (9H, m), 6.92-6.79 (4H, m), 6.28-6.20 (1H
unresolved m), 4.69 (1H, m), 4.45 (1H, m), 4.0-3.2
20 (12H, m), 2.80 (1H, m), 2.69 (2H, t), 2.32 (1H, m),
1.15 (12H, m); ^{13}C NMR 400MHz ($CDCl_3$, ppm) δ 20.3 (m),
24.6 (m), 43.2 (m), 55.2, 58.3 (d), 64.1 (d), 75.8
(d), 76.0 (d), 77.9, 85.6 (d), 86.3, 113.2, 117.7 (d),
122.8 (d), 123.1 (d), 124.8 (d), 125.1, 125.2, 125.9,
25 126.8, 127.1, 127.5, 127.6 (d), 127.8 (d), 127.9,
128.4, 130.3, 130.7 (d), 131.4, 135.4, 136.1 (d),
145.1, 158.5; HRMS (FAB, 3-NBA matrix) calculated for
 $C_{51}H_{54}N_2O_6P$ (M+H) 821.3722, found 821.3720.

30 2 DMT phosphoramidite (280 mg, 77%): 1H NMR
($CDCl_3$, ppm), 8.78 (1H, d, $J=8.0$), 8.68 (1H, d,

1 J=8.0), 8.07 (2H, m), 7.8 (1H, d, J=8.0), 7.80-7.24
(12H, m), 6.84 (4H, overlapped d, J=8.0), 5.94 (1H,
overlapped dd, J=2.9,10), 4.62 (1H, m), 4.40 (1H, m),
3.9 (2H, m), 3.8 (6H, s), 3.50 (2H, m), 3.83 (3H, s),
5 3.77 (3H, s), 3.66-3.45 (3H, m), 3.42-3.38 (2H, m),
2.82, (2H, t, J=5.6), 2.52 (3H, t, J=5.6), 2.16 (1H,
m), 1.20-1.05, (12H, m); ^{13}C NMR 400MHz (CDCl_3 , ppm) δ
20.0 (m), 24.5 (m), 42.0, 43.2 (m), 55.1, 58.4 (m),
63.9 (d), 75.3 (d), 75.8 (d), 77.2 (obscured by
solvent), 85.0 (m), 86.2, 113.1, 117.5, 122.4, 123.0
10 (d), 123.2 (d), 124.1 (d), 126.2, 126.4 (d), 126.6
(d), 126.7 (d), 127.8, 128.3 (d), 128.8 (d), 129.7
(d), 129.9, 130.1 (d), 130.6, 131.6, 136.1 (m), 144.9,
158.4; HRMS (FAB, 3-NBA matrix) calculated for
 $\text{C}_{49}\text{H}_{53}\text{N}_2\text{O}_6\text{PNa}$ 819.3537, found 819.3539.

15 **3** DMT phosphoramidite (48 mg, 50%): ^1H NMR
(CDCl_3 , ppm) δ 8.10 (1H, d, J=8.0), 7.9 (1H, d,
J=8.0), 7.8 (1H, d, J=8.0), 7.52-7.24 (9H, m), 6.82
(4H, overlapped d, J=8.0), 5.91 (1H, overlapped dd, 2
x isomers), 4.6 (1H, m), 4.38 (1H, m), 3.83 (3H, s),
20 3.77 (3H, s), 3.66-3.45 (3H, m), 3.42-3.38 (2H, m),
2.82 (2H, t, J=5.6), 2.52 (3H, t, J=5.6), 2.16
(1H, m), 1.20-1.05, (12H, m); ^{13}C NMR, 400MHz (CDCl_3 ,
ppm) δ 20.3 (m), 24.5 (m), 42.0, 43.1 (m), 55.1, 58.4
(m), 63.9 (d), 75.1 (d), 76.2 (d), 77.1 (obscured by
25 solvent), 85.0 (m), 86.1, 113.0, 122.3 (d), 123.4 (d),
125.4, 125.6 (d), 126.0 (d), 126.8 (d), 127.7, 128.2
(d), 128.6 (d), 130.1 (d), 130.5 (d), 133.6 (d), 136.0
(d), 137.5, 144.9, 158.4; HRMS (FAB, 3-NBA matrix)
calculated for $\text{C}_{45}\text{H}_{52}\text{N}_2\text{O}_6\text{P}$ (M+H) 747.3565, found
30 747.3563.

1 **4** DMT phosphoramidite (170 mg, 65%): ^1H NMR
(CDCl_3 , ppm) δ 7.91 (1H, s), 7.83-7.79 (4H, m), 7.56-
7.27 (11H, m), 6.87 (4H, overlapped d, $J=8.0$), 5.39
(1H, dd, $J=2.9$, 10), 4.58 (1H, m), 4.30 (1H, m), 3.90
5 (2H, m), 3.83 (3H, s), 3.77 (3H, s), 3.66-3.45 (3H,
m), 3.42-3.38 (2H, m), 2.82 (2H, t, $J=5.6$), 2.52 (3H,
t, $J=5.6$), 2.16 (1H, m), 1.20-1.05 (12H, m)

5 DMT phosphoramidite (380 mg, 89%): ^1H NMR
(CDCl_3 , ppm) δ 7.54 (2H, 5 overlapped d, $J=8.0$), 7.45-
7.40 (4H, m), 7.36-7.22 (3H, m), 6.98 (1H, s), 6.90-
10 6.82, (4H, m), 5.37 (1H, dd, $J=2.9$, 10), 4.56 (1H, m),
4.22 (1H, m), 3.82 (6H, s), 3.66-3.45 (3H, m), 3.42-
3.38 (2H, m), 2.82 (2H, t, $J=5.6$), 2.48 (1H, m), 2.29,
(3H, s), 2.24 (3H, s), 2.18 (3H, s), 1.99 (1H, m),
15 1.28 (1H, m), 1.20-1.05 (12H m); ^{31}P NMR (CDCl_3 , ppm) δ
148.9, 148.4; HRMS (FAB, 3-NBA matrix) calculated for
 $\text{C}_{44}\text{H}_{55}\text{N}_2\text{O}_6\text{P}$ 739.3876, found 739.3870.

6 DMT phosphoramidite (420 mg, 84%): ^1H NMR
(CDCl_3 , ppm) δ 7.51 (2H, m), 7.42-7.22 (9H, m), 6.84
(4H, overlapped d, $J=8.0$), 6.78 (1H, dd, $J=9.0$, 8-6),
20 5.38 (1H, dd, $J=2.9$, 10), 4.54, (1H, m), 4.22 (1H, m),
3.82 (6H, s), 3.66-3.45 (3H, m), 3.42-3.38 (2H, m),
2.82 (2H, t, $J=5.6$), 2.50, (2H, t $J=5.6$), 2.05 (1H,
m), 1.20- 1.05 (12H, m); ^{31}P NMR (CDCl_3 , ppm) δ 148.9,
148.3; HRMS (FAB, 3-NBA matrix) calculated for
25 $\text{C}_{42}\text{H}_{49}\text{F}_2\text{N}_2\text{O}_6\text{P}$ 769.3194, found 769.3209.

30

35

As seen in reactions with benzene derivatives,¹³
1 coupling with the larger polycyclic aromatics yields a
mixture of alpha- and beta-anomers in good overall
yields (54-81% isolated yields). The major isomer in
all four cases is formed with retention of
5 configuration; thus, alpha-anomeric C-nucleosides (the
p-toluoyl esters of 1a, 2a, 3a, 4a of Fig. 5) are the
primary products. Measured ratios of the two isomers
(by NMR integration) ranged from 5:1 ($\alpha:\beta$) for the 1-
naphthyl derivative to 10:1 for the 9-phenanthrenyl
10 derivative. The configuration at the C-1' carbons of
all isomers was determined by analysis of H1'-H2'
coupling constants for the protected nucleosides, by
¹H-nuclear Overhauser experiments on the deprotected
nucleosides, and by correlation with an x-ray crystal
15 structure of one of the α -anomeric compounds (see
below).

Although the desired beta-anomers (the toluoyl
esters of 1-4, Fig. 5) could also be isolated from
this coupling reaction, the yields were less than
20 ideal. Studies were thus undertaken to find
conditions under which the predominant alpha-anomers
could be converted to the desired beta- configuration.
It was anticipated that acidic conditions might allow
epimerization at the C-1' position by reversible ring-
25 opening. Experimentation with several sets of
conditions revealed that benzenesulfonic acid in
reflexing xylene, in the presence of a small amount of
water, did indeed result in ready equilibration of the
alpha-anomers to mixtures of beta- and alpha-isomers
30 after several hours. Addition of a small amount of

1 water was found to be necessary for the isomerization.
The equilibration was then carried out for all four
alpha-isomers (1a, 2a, 3a, 4a of Fig. 5) as their bis-
toluoyl esters. The isomerization was also tested on
5 two previously reported substituted benzene
nucleosides (the toluoyl esters 5a, 6a) to test the
scope of the reaction. Significantly, the major
component of each mixture after equilibration was in
all cases the desired beta-anomer (1-6 of Fig. 5).
The ratios of beta-to alpha-isomers ranged from 4:1
10 for the trimethylbenzene derivative to 2.5:1 for the
1-naphthyl derivative. Isolated yields of the desired
beta-anomers after column chromatography ranged from
28-54%. The alpha anomers and mixed fractions could
be reisolated and recycled in the isomerization if
15 desired. Interestingly, the deprotected free
nucleosides themselves did not undergo any observable
isomerization under these conditions, even at extended
reaction times.

20 With the new method for epimerization to beta-
anomeric configuration in hand, the synthetic scheme
made possible the facile generation of the six
aromatic C-nucleosides (1-6 of Fig. 5) in generally
good yields (Fig. 7). The toluoyl protecting groups
were removed in methanolic base with yields ranging
25 from 50-78%. Following this overall scheme, the free
unprotected nucleosides were produced in a total of
only three steps (aromatic coupling, isomerization,
ester deprotection).

30

35

EXAMPLE 2

1

Structural assignments

The structural assignment of anomeric configuration for compounds 1-6 and 1a-6a (Fig. 5) was made by ¹H-NOE studies of all compounds, by
5 examination of coupling constants for H1' and H2' protons, and by correlation with an x-ray crystal structure of the alpha-1-naphthyl nucleoside 3a (Fig. 2). In addition, the compounds were characterized by their ¹H and ¹³CNMR spectra and by high-resolution mass
10 spectrometry.

¹H, ¹³C, and ³¹P NMR spectra were recorded with a 300 MHz spectrometer unless otherwise noted, and chemical shifts are given in δ (ppm) using solvent as
15 internal reference, and the coupling constants are in Hertz (Hz). NOE difference spectral were also performed on a 300 MHz instrument. The mass spectra were performed using electron impact or chemical
20 ionization. All reactions were monitored by thin-layer chromatography (TLC) using EM Reagents plates with fluorescence indicator (SiO₂-60, F-254). Flash column chromatography was conducted using EM Science Silica Gel 60 (230-400 mesh). Mass spectral analyses were performed by the University of California,
25 Riverside Mass Spectrometry Facility, Riverside, CA. All reactions were carried out under a nitrogen atmosphere in dry, freshly distilled solvents under anhydrous conditions unless otherwise specified. The
30 was distilled from sodium metal/benzophenone, methylene chloride was distilled from NaH, and pyridine was distilled from BaO prior to use.

35

1 Proton nuclear Overhauser effects were used to
examine the geometries of the anomeric isomers of
compounds 1-6 (Fig. 5) The approach used was to
separately irradiate the H-2' proton resonances
situated at δ 1.7-2.7 ppm and observe enhancements at
5 vicinal 1' and 3' protons (see Figure 1 and Tables
1,2). Although specific assignment of which resonance
corresponds to 2'- α and which to 2'- β could not be
made *a priori*, analysis of predicted NOE effects makes
possible a simple approach to assignment of
10 stereochemistry at the 1' position. Examination of
the structures of alpha- and beta- nucleosides (Figure
1) shows that for alpha anomers, the 2'- β proton is in
close proximity to both the 1' and 3' protons, while
the C-2'- α proton is not near either one of these
15 protons. In beta-anomers, on the other hand, the 2'- β
proton is near only the 3' proton, while the 2'- α
proton is only near the 1' proton. Thus, in an alpha
anomer, separate irradiation of each of the C-2'
protons should lead to two and zero NOE enhancements
20 at the vicinal protons, while in a beta anomer, these
two irradiations would lead to one significant
enhancement for each irradiation.

To test this prediction, experiments on naphthyl
nucleoside isomers 3 and 3a (Tables 1 and 2) were
25 carried out. The diester of 3a is the principal
product of the glycosidic coupling reaction (with the
diester of 3 being a minor product). Irradiation of
one of the 2' protons of the nucleoside gave
significant nuclear Overhauser enhancements of 8% and
30 7% at the 1' and 3' protons; however, irradiation of

1 the other 2' proton gave no significant enhancement at
either 1' or 3' positions. Using the analysis above,
this indicates that this compound is an alpha anomer.
This assignment was confirmed by a single-crystal x-
ray structure obtained for the compound (below). To
5 complete the analysis of the two isomers we carried
out the same experiments on the isomeric nucleoside 3,
which is the major product after epimerization of 3a.
Irradiation of one of the 2' protons gave an 8%
enhancement of the 1' proton (and none at the 3'
10 proton), while irradiation of the other 2' proton gave
a 5% enhancement of the 3' proton (and none for the 1'
proton). Thus, this compound is assigned to the beta
configuration. Also consistent with this assignment
is a separate experiment in which the 1' proton was
15 irradiated; here we observed 6% enhancement at the 4'
proton (Table 1), while the alpha isomer shows no such
enhancement (data not shown).

The NOE experiments were then carried out for
the isomers of 1, 3, and 4-6 (Fig. 5). The results
20 are shown in Tables 1 and 2. The results were all
consistent with the model, in that one isomer of each
pair gave two and -zero enhancements of the vicinal
protons for the two H-2' irradiations, while the other
clearly gave one significant enhancement for each of
25 the two irradiations. The isomers which gave two and
zero enhancements were assigned as alpha-anomers, and
those with one and one enhancements were assigned to
be beta-anomers. Also consistent with these
assignments were NOE enhancements in the H-4'
30 positions on irradiation of the H-1' protons for the

1 beta isomers (Table 1) which were absent in the alpha
isomers.

These assignments were also internally
consistent in that the major isomers obtained from the
glycosidic coupling reaction were all of the same
5 anomeric configuration (alpha). Similarly, the major
isomers isolated from the epimerization were all of
the same anomeric configuration (beta). In addition,
all the isomers assigned as alpha had H-1' resonances
which qualitatively appeared as pseudo-triplets (they
10 are actually doublets of doublets), having both
coupling constants near 6.0-8.0 Hz. The isomers
assigned to the beta configuration had H-1' resonances
which appeared as nearly evenly spaced doublets of
doublets (J~ 3 and 10 Hz). These H-1' - H-2' coupling
15 constant trends are consistent with a literature
report of similar coupling constants for related β -C-
nucleoside¹⁸ (although they are reversed relative to
observations for β -N-nucleosides).

Also useful in confirmation of these structural
20 assignments was x-ray structural data obtained for 1-
naphthyl compound 3a (Figure 2). A crystal suitable
for analysis was obtained by recrystallization from
methylene chloride/hexane (CH₂Cl₂/hxane).

Measurements were made on an Enraf-Nonius
25 diffractometer with graphite monochromated Mo-K α
radiation. Single crystals of C₁₅H₁₆O₃ are monoclinic,
space group P2₁ (#4), with $a=7.806(2)$ Å, $b=6.720(2)$ Å,
and $c=11.898(3)$ Å, $V=615.8(3)$ Å³, $Z=2$ with calculated
density 1.31g/cm³. The data was collected at -20(1)°C
30 using the $\omega/2\theta$ scan technique to a maximum 2θ of

50.0°. Omega scans of several intense reflections,
1 made prior to data collection, had an average width at
half-height of 0.26° with a take-off angle of 2.8°.
The counter temperature consisted of a variable
horizontal slit with a width ranging from 2.0 to 2.5
5 mm and a vertical slit set to 2.0 mm. The diameter of
the incident beam collimator was 0.7 mm and the
crystal to detector distance was 21 cm. A total of
1234 unique absorption-corrected reflections were
10 collected, and the structure was solved by direct
methods. The non-hydrogen atoms were refined
anisotropically, and the hydrogen atoms were included
in idealized positions. The final cycle of full-
matrix least-square refinement was based on 785
15 observed reflections and converged (largest parameter
shift was 0.01 times its esd) with unweighted and
weighted agreement parameters of $R=0.043$ and $R_w=0.039$.
The standard deviation of an observation of unit
weight was 1.54. The maximum and minimum peaks on the
20 final difference Fourier map corresponded to 0.14 and
-0.15 e-/Å³, respectively. All calculations were
performed with the teXsan software package of the
Molecular Structure Corporation.

The structure shows the α -configuration and in
analogy to natural nucleosides, the naphthalene is in
25 an anti- conformation, with the aromatic group
oriented away from the sugar. The deoxyribose ring is
in a C-3'-exo (S-type) conformation.

Experimental H'-1' to H-2' coupling constants
for the ester of this compound (3a) in CDCl₃ were
30 $J=8.0$ and 6.0 Hz. The corresponding dihedral angles

1 generated from the x-ray structure are found to be
8.1° and 124.5°. Application of the Karplus
relationship empirically adjusted for nucleosides²⁰
predicts J=9.2 and 2.8 Hz, respectively, indicating a
5 small change in ring conformation in solution relative
to that in the crystal (or non-ideal match of the
empirical relationship of this C-nucleoside).
Interestingly, although this compound clearly is an
alpha-anomer, the experimentally measured coupling
10 constants are more consistent with those commonly seen
for beta-, rather than alpha-, anomers of natural
nucleosides.

15

20

25

30

35

EXAMPLE 3

1

Incorporation into DNA

The β -C-deoxynucleosides 1, 2, and 3 (of Fig. 5) were then carried on with the aim of incorporating them into DNA oligonucleotides by automated solid-phase methods (Fig. 7 and Figs. 14-15). Standard methods were used to convert the unprotected nucleosides to 5'-dimethoxytrityl-protected derivatives in yields ranging from 59-92% after purification. These were then converted into cyanoethyl phosphoramidite derivatives, which were obtained in 50-89% yields after purification by column chromatography.

DNA oligonucleotides were synthesized on an Applied Biosystems 392 synthesizer using standard β -cyanoethylphosphoramidite chemistry but with extended (10 Minute) coupling cycles for the normal residues. Stepwise coupling yields for the nonnatural residues were all greater than 90% as determined by trityl cation monitoring. Oligomers were purified by preparative 20% denaturing polyacrylamide gel electrophoresis and isolated by the crush and soak method, and were quantitated by absorbance at 260 nm. Molar extinction coefficients were calculated by the nearest neighbor method. Values for oligonucleotides containing nonnatural residues were estimated the following way: each of the new nucleotides was measured for its excitation coefficient at 260 nm. The molar extinction coefficients for 2 and 3 were found to be 8990, and 154, respectively, and these values were added to the value for the core sequence dCGCGCG.

35

1 The absorbance of nucleoside 1 in DNA was
measured at 350nm and 0.48 of this value was
subtracted from the total absorbance at 260nm to get
the absorbance of the core DNA alone.
5 Oligodeoxynucleotides were obtained after purification
as the sodium salt. Intact incorporation of residues
1-3 was confirmed by synthesis of short oligomers of
sequence T-X-T (where X=1-3). Proton NMR (400 MHz)
(Fig. 3) indicated the presence of the intact
structures with the expected integration.

10 The spectra of the crude unpurified
oligonucleotides show clear resonances very similar to
those of the free nucleosides, and having the expected
aromatic integrations relative to anomeric C-1'
protons and thymine C-6 protons and C-5 methyl groups.
15 This confirms both the presence of the intact
structures (as expected for unreactive aromatic
hydrocarbons) as well as the high coupling yields,
since di- and mononucleotides which result from
incomplete coupling are not seen.

20

25

30

35

EXAMPLE 4

1

Fluorescence properties in DNA

Since polycyclic aromatics such those in the
nucleosides 1, 2, and 3 (Fig. 5) have been studied in
5 other contexts as fluorescent probes,²² the
fluorescence properties of oligonucleotides containing
these structures in aqueous buffer was examined.
Heptamer oligodeoxynucleotides having the sequence 5'-
dXCGCGCG, (where X=1, 2 and 3) which are self-
10 complementary and form duplexes with the polycyclic
aromatic nucleoside situated at the 5' ends were
synthesized. These were purified by preparative
denaturing gel electrophoresis.

Fluorescence spectra were recorded on a SPEX-
15 Fluorolog-2 series fluorometer. A xenon lamp was used
as the source of radiation. The fluorescence
measurements were taken in the right angle mode using
0.1-0.15 μ M DNA solutions in a pH 7.0 buffer (10mM Na-
PIPES, 10mM MgCl₂, 100mM NaCl). Five scans were
20 averaged at 23°C. The excitation slits were set to
6mm and the emission slits to 2mm. All emission
spectra were corrected using a reference dye
(rhodamine-B) to compensate for instrument
fluctuations, and also by subtraction of data for
25 buffer alone. Excitation wavelengths of 233, 251 and
341 nm (the absorbance maxima) were used to excite the
compounds containing naphthalene, phenanthrene and
pyrene, respectively.

30

35

1 Emission spectra were measured for the three
sequences in a pH 7.0 buffer (10mM PIPES buffer, 100
mM NaCl, 10mM MgCl₂) at 25°C, conditions under which
they likely form duplexes. The naphthalene-containing
5 sequence showed no emission detectable above
background. The other compounds showed fluorescence
emission profiles consistent with published spectra
for the polycyclic aromatic parent structures.²² The
phenanthrene-containing oligonucleotide had the most
10 intense emission (Figure 4), with the strongest peak
at 370 nm. The pyrene-modified sequence showed a
similar emission profile but with an emission maximum
at 395 nm and with peak intensity considerably lower
than that for the phenanthrene case, suggesting
15 considerable quenching by the DNA under these
conditions and in this sequence.

20

25

30

35

EXAMPLE 51 **Properties of the α -pyrene nucleoside in DNA**

5 The α -pyrene nucleoside phosphoramidite was incorporated into DNA on an ABI 392 synthesizer on 1-
10 μ mole scale. Coupling yields were monitored by trityl response and were high (-92% or greater). Several oligonucleotides (Figure 13) were prepared which were designed to form triplexes with a single-stranded target by folding back. The molecules have five-
15 nucleotide loops composed either of thymine nucleotides or with varying numbers (1, 3, 5) of pyrene nucleotides. These were synthesized without complication and purified by denaturing preparative PAGE gels.

20 Thermal denaturation experiments were performed as described previously, using a heating rate of 0.5°C/min. Solutions for the thermal denaturation studies contained a 1:1 molar ratio of oligonucleotide probe and its corresponding complementary 18-nt
25 oligomer (1.5 μ M each). Solutions were buffered with 10mM Na•PIPES (1,4-piperazine-bis(ethanesulfonate), Sigma) at pH 7.0. Also present in the denaturation solutions were 100 mM NaCl and 10 mM MgCl₂. After the solutions were prepared they were heated to 90°C and
30 allowed to cool slowly to room temperature prior to the melting experiments. Uncertainty in T_m is estimated at \pm 0.5°C based on repetitions of experiments.

35 The results of binding studies (thermal denaturation) showed that all oligomers bound the

1 single-stranded target well; thus, the new nucleoside
does not interfere with binding properties. Separate
binding studies were also carried out using longer
targets which extend beyond the loop; binding was
still tight, and interestingly, the pyrene-tagged
5 compounds showed stronger binding the more pyrenes
were present. For example, a complex with the P1
oligomer had a T_m of 35°C at pH 7.0, while the P3
probe bound with a T_m of 42°C, or 7°C higher. Binding
to the sequence shown above was also demonstrated by
10 native gel shift experiments, and the complexes were
clearly visible under fluorescent light. While the
single-pyrene version was deep blue in color, the
multi-tagged ones were light blue to white in
appearance, indicative of longer-wavelength emission.
15 See Fig. 13 for description of P oligonucleotides.

Fluorescence properties

Steady-state fluorescence spectra were recorded
on a SPEX-Fluorolog-2 series fluorometer at room
20 temperature. The source of radiation was a xenon arc
lamp. An excitation wavelength of 341 nm was used,
and all excitation and emission slits were set to 2
mm. Fluorescence measurements were taken in the right
angle mode. All sample concentrations were 0.1 μ M in
25 labeled DNA in a pH 7.0 buffer (10 mM Na•PIPES, 10 mM
MgCl₂, 100mM NaCl). The buffer solutions were air-
saturated. For the duplex measurements, an equimolar
amount of the Watson-Crick complement was added, the
mixture heated to 90°C and slow cooled before
30 remeasuring the fluorescence. All emission spectra

1 were measured using a reference dye (rhodamine-B) to
compensate for lamp fluctuations and were corrected by
subtraction of data for buffer alone unless indicated
otherwise. 1-Pyrenemethylamine hydrochloride was
obtained from Alrich.

5 The singly-tagged oligonucleotide (P1) of Fig.
13 was studied first. The spectrum of oligonucleotide
P1 was compared to that of the free nucleoside by
itself in water at the same concentration (0.1 μ M).
10 The compounds were excited at 341 nm and studied in a
pH 7.0 buffer (100 mM NaCl, 10 mM MgCl₂, 10 mM Na-
PIPES). The spectra are shown in Figures 9 and 10.

15 The free nucleoside shows an emission spectrum
very similar to that published for pyrene, with the
most intense peak at about 400 nm. The P1
oligonucleotide at the same concentration shows an
emission spectrum with less structure and with a tail
out to longer wavelengths. Interestingly, the
emission intensity of P1 compound is 500,000 at 381
20 nm, while that of the free nucleoside in water is
50,000. Thus, incorporation of the nucleoside into a
DNA strand leads to an increase in emission intensity
of about 10 fold.

25 The multi-tagged oligonucleotides have two
unusual characteristics when examined by fluorescence
spectroscopy. First, the emission spectra show a
clear excimer band at about 450-550 nm (peak at 483
nm). Second, the intensity of the excimer band is
more intense than the normal pyrene fluorescence of P1
for the two compounds with three and five labels.
30 Interestingly, when three labels were placed with

1 thymines between (comparing P3 and P3A), the excimer
band is much weaker, confirming that excimer requires
adjacent placement of the labels. Three pyrenes in a
loop gave a stronger excimer band than did three at
the end (compare P3 to P3END), indicating greater
5 stacking in the loop (Figure 13).

All five fluorescent-tagged oligomers were then
hybridized to the complementary target DNA.
Importantly, the fluorescent emission intensity stayed
the same (within 20%) on binding. This compares very
10 favorably to pyrenebutyrate linked to DNA, which is
quenched by about 10 fold on binding a complement^{30b}.

The P3 oligonucleotide was dimerized to circular
form following the procedure of Rubin et al. 1995
Nucleic Acids Res. 23 :3547-3553. This
15 oligonucleotide binds quite tightly and specifically
to a 20-nt recognition site in DNA. The emission
intensities of the P3 oligomer (containing 3 pyrene
nucleosides) were compared to that of the circular P6
oligomer (containing 6 pyrene nucleosides) under
20 identical conditions (See Figure 13).

Results show that the P6 compound has an
emission intensity twice that if the P3
oligonucleotide (see Figure 12). Thus, six pyrene
labels are present in this DNA probe with no quenching
25 of fluorescence intensity. The emission intensity of
this P6 circle at 483 nm is about four times that of
the singly-labeled P1 compound (emission maximum 381
nm), and about forty times than that of the free-
pyrene nucleoside in the same buffer.

30

35

1 These results show that multiple labels can be
incorporated into DNA, yielding emission which can be
considerably brighter than a single label. In
addition, the emission is at longer wavelengths which
are more easily visible.

5

10

15

20

25

30

35

EXAMPLE 6

1 **Substitution in linear oligonucleotide probes**

5 Experiments were performed to optimize multiple
 alpha-pyrene substitution at the ends of standard DNA
 probes rather than substitution in loop regions of
 triplex-forming probes as in Example 5. First
 evaluated was whether 5'-end or 3'-end substitution
 was favored, and whether the neighboring base
 mattered. Experiments were carried out with oligos
 10 containing 3 adjacent alpha-pyrenes; these were
 observed for the florescence emission intensity both
 unbound and when hybridized. The probes are designed
 to bind to human telomere repeats. Results are shown
 below for unbound oligomers (the relative results do
 15 not change when they are bound).

<u>sequence</u>	<u>rel intensity(485 nm)</u>
5'-CCC TAA CCC TAA CCC TAA <u>PPP</u> -3'	1.0 x 10 ⁷
5'- <u>PPP</u> CCC TAA CCC TAA CCC TAA-3'	4.4 x 10 ⁶
20 5'- <u>PPP</u> AAC CCT AAC CCT AAC CCT-3'	1.0 x 10 ⁷

25 Results show that 5' end and 3' end substitution
 give identical fluorecence intensity. The nature of
 the adjacent base, however, does make a difference.
 Comparison of the cases when the adjacent base is A or
 C shows that the case with A adjacent to a pyrene gave
 significantly (50% higher) emission intensity.

30 Next, how best to add additional pyrenes for
 greater intensity was examined. Results with pyrenes
 substituted internally in DNA (Example 5) indicated

1 that emission intensity for the excimer increases with
 1 three to five adjacent pyrenes, but adding another
 (six adjacent labels) actually decreases overall
 intensity. A similar effect is seen with pyrenes
 incorporated at the end of a linear probe.

5 Three oligos having multiple pyrenes were
 compared: one with three adjacent labels (P3), one
 with six adjacent labels (P6), and one with three
 labels, then an adenine, then another three labels
 (P3A3). Of these, P3A3 gave much better intensity
 10 than the other two. Thus, yet brighter labels may be
 possible by using configurations such as P3AP3AP3, or
 P5AP5, or adding such groups at both ends of the
 probe.

15	<u>sequence</u>	<u>rel intensity(485 nm)</u>
	5'-CCC TAA CCC TAA CCC TAA <u>PPP</u> -3'	3.8 x 10 ⁶
	5'-CCC TAA CCC TAA CCC TAA <u>PPP</u> A <u>PPP</u> -3'	7.2 x 10 ⁶
	5'-CCC TAA CCC TAA CCC TAA <u>PPPPPP</u> -3'	1.1 x 10 ⁶

20

25

30

35

1

EXAMPLE 7**Comparison of excimer fluorescence properties
of the two isomers**

5 The same DNA sequence described in Example 5 was
synthesized with three consecutive pyrene residues;
one sequence had three alpha isomers, and the other,
three beta isomers. Fluorescence emission was
measured for both with the same excitation and same
10 solution conditions. Results show that both
oligonucleotides show virtually the same emission
bands consistent with full excimer formation with loss
of pyrene fluorescence. The peak height for the alpha
case however, is ~100 times higher than for the beta
15 case (see Figure 17.)

 There is likely to be very little inherent
fluorescence difference for the two chromophores
alone. The likely reason for the large difference in
DNA is that the beta case (which has the same
20 configuration as natural DNA bases) stacks more
strongly with neighboring DNA, and that this stacking
leads to stronger quenching by neighboring bases.

25

30

35

EXAMPLE 8

1

Further studies were performed on the following oligonucleotides:

	<u>Name</u>	<u># Pyrenes</u>	<u>Sequence</u>
5	HT0	0	5' -CCC TAA CCC TAA CCC TAA
	HT1	1	5' -CCC TAA CCC TAA CCC TAA P
	HT2	2	5' -CCC TAA CCC TAA CCC TAA PP
	HT3	3	5' -CCC TAA CCC TAA CCC TAA PPP
10	HT4	4	5' -CCC TAA CCC TAA CCC TAA PPPP
	HT5	5	5' -CCC TAA CCC TAA CCC TAA PPPPP
	HT6	6	5' -CCC TAA CCC TAA CCC TAA PPPPPP
	HT3A3	6	5' -CCC TAA CCC TAA CCC TAA PPPAPPP
	HT7	7	5' -CCC TAA CCC TAA CCC TAA PPPPPPP
15	HT3B	3	5' -PPP AAC CCT AAC CCT AAC CCT
	HT4B	4	5' -PPPP AAC CCT AAC CCT AAC CCT
	HT5B	5	5' -PPPPP AAC CCT AAC CCT AAC CCT
	HT6B	6	5' -PPPPPP AAC CCT AAC CCT AAC CCT
	HT3C	3	5' -PPP CCC TAA CCC TAA CCC TAA
20	tel3	0	5' -TTA GGG TTA GGG TTA GGG
	tel3B	0	5' -AGG GTT AGG GTT AGG GTT

DNA oligonucleotides were synthesized on an Applied Biosystems 392 synthesizer using standard β -
 25 cyanoethylphosphoramidite chemistry but with extended (12.5 minute) coupling cycles for the pyrene residues, as described.^{31,32} Stepwise coupling yields for the pyrene residues were typically greater than 95% as determined by trityl cation monitoring. A pyrene
 30 phosphoramidite concentration of 0.05M in acetonitrile

35

1 was used, and shown not to compromise coupling
efficiency. DNA oligomers were purified by
preparative 20% denaturing polyacrylamide gel
electrophoresis and isolated by the crush and soak
5 method. Molar extinction coefficients of unmodified
oligonucleotides were calculated by the nearest
neighbor method.³³ Concentrations of oligonucleotides
containing pyrene residues were determined in the
following way: UV absorbances were measured at 260 nm
10 and 350 nm. The 260 nm values were substituted into
Beer's Law, using the calculated extinction
coefficient for the DNA portion of the
oligonucleotide. A correction factor for pyrene's
contribution at 260 nm was taken to be 0.5 times the
15 absorbance at 350 nm. This gave an approximate
concentration of the labeled oligomer. To reach a
more accurate oligomer concentration, Job's plots were
constructed from mixing experiment data carried out
with the Watson-Crick complement to the
oligonucleotides.

20
Thermal denaturation experiments. Solutions for the
thermal denaturation studies contained a 1:1 molar
ratio of oligonucleotide probe and its corresponding
complementary 18-nt oligomer (1.5 μ M each). Solutions
25 were buffered with 10 mM Na·PIPES (1,4-piperazine-
bis(ethanesulfonate), Sigma) at pH 7.0. Also present
in the denaturation solutions were 100 mM NaCl and 10
mM MgCl₂. After the solutions were prepared they were
heated to 90°C and allowed to cool slowly to room
30 temperature prior to the melting experiments. The

35

1 melting studies were carried out as described
previously,¹⁴ using a heating rate of 0.5°C/min.
Uncertainty in T_m is estimated at $\pm 0.5^\circ\text{C}$ based on
repetitions of experiments.

5 **Fluorescence of single pyrene nucleosides in solution
and in DNA.** Fluorescence measurements were performed
as described in Example 5. The 18mer DNA sequences
chosen for labeling are complementary to the human
10 telomere repeat sequence.³⁴ The fluorescence emission
spectra for the free nucleosides of alpha- and beta-
pyrene were first measured. They were sparingly
soluble in water and so were measured in methanol
(Fig. 19A). They were found to have identical
15 emission profiles and intensities, and show three
bands between 350-425nm ($\lambda_{\text{max}}=396\text{nm}$), typical of pyrene
monomer emission. A single α -pyrene label was
incorporated at the oligonucleotide 3' end (sequence
HT1, with pyrene adjacent to adenine) and the
20 fluorescence spectrum measured in aqueous buffer (Fig.
19B). The emission profile was found to be
essentially identical to that of the free nucleoside
and to that of pyrenemethylamine hydrochloride (PMAH).
Using PMAH as a model for free pyrene in aqueous
25 buffer, comparison of emission intensities at equal
pyrene concentrations shows a ~17-fold decrease in
intensity upon incorporation of α -pyrene into this DNA
sequence, consistent with previous observations.^{30b}

30 **Multiple pyrene substitution in oligodeoxynucleotides.**
Preliminary studies comparing alpha- and beta-pyrene

35

1 labels in the same sequence of DNA showed consistently
that the beta-pyrene labels gave much lower
fluorescence intensity, despite the fact that as free
nucleosides the two behave identically. One example
5 of this effect is shown in Fig. 20, which compares
emission spectra for a sequence containing three
consecutive alpha- or beta- labels. Although emission
profiles are virtually the same, with the beta
configuration the intensity is about 100 times lower.

10 A series of oligonucleotides carrying 1-7 α -
pyrenes at the 3' end (sequences HT1-HT7) and 3-6 α -
pyrenes at the 5' end (HT3B-HT6B) was synthesized.
The emission spectra are shown for the 3' series in
Fig. 21, and the absorption spectra in Fig. 22. The
15 numerical data are given in Table 3. For all cases,
when two or more adjacent pyrenes are present in DNA,
excimer formation was observed as a broad
structureless band between 400 and 600 nm (peak
maximum ~489 nm) (Figure 21A). With two pyrenes there
is a very small amount of residual monomer emission
20 seen (excimer/monomer is >20:1), and beyond three
labels (Fig. 21B) no monomer emission is detectable.
Similar results are observed at the 5' end of the
sequence (see Table 3), although for those cases a
low-intensity shorter wavelength shoulder was visible
25 on the excimer band at ca. 425-450nm.

The emission intensity trends were observed to
be generally similar at both ends of the DNA if the
neighboring base is the same (Table 3). For both
series the emission intensity increases generally and
30 reaches a peak at five labels, after which further

1 increasing the number of consecutive labels actually
results in a decrease in overall emission intensity
(Fig. 21, Table 3). Interestingly, the relative
emission intensities correlate well with the rank
5 order of molar absorptivities of each labeled oligomer
(Fig. 22, Table 3). For example, the sequence with
six α -pyrenes actually has both a lower molar
absorptivity (by nearly 300-fold at 348nm) and a lower
fluorescence emission intensity (by 21-fold) than that
10 with five, despite the greater number of pyrenes. For
the HT2-7 series excitation spectra was also measured
by monitoring at 489 nm, and the resulting spectra
were virtually identical to the absorption spectra in
Fig. 22.

15 **Even/odd effects in emission intensity.** Viewing the
series as a whole, an interesting trend is seen with
the number of adjacent pyrenes and the intensity of
excimer formation (Fig. 23). Not unexpectedly, three
pyrenes are more brightly fluorescent than two, but
20 surprisingly, four are not as bright as three. Five
pyrenes yields very bright emission, while six is much
less intense. Seven labels again shows a small
increase in intensity. In general, an odd number of
pyrenes seems to favor more intense excimer emission,
25 whereas an even number produces weak emission (Figure
21B, Table 3). The same trend was observed for both
the 3' and 5' labeled oligomer series. This is
further substantiated by examining the fluorescence
intensity for two different oligomers each possessing
30 six pyrenes. The one with six adjacent pyrenes is

1 much less fluorescent than the one where they are
divided into two groups of three with an adenine in
between (HT6 vs. HT3A3, Table 3). Examination of the
absorption spectra (Fig. 22) shows that the rank order
of absorptivity correlates well with emission
5 intensity. Thus, the even/odd effects are seen at the
ground state prior to excitation.

Evidence for preassociation. To determine whether the
excimer fluorescence arises from excited-state or
10 ground-state association of pyrenes in these multi-
labeled DNAs, several pieces of data was examined.
First, measurement of absorption spectra for free
pyrene (PMAH) and alpha-pyrenes in DNA (sequences HT1-
7) shows that all the DNA-associated pyrenes exhibit a
15 redshift of ~6-9nm. Free PMAH in buffer has peak
maxima at 325 and 342 nm, whereas those of HT1-7 lie
at ~331 and 348nm (Table 3), consistent with stacked
species. Second, observation of absorption band
broadening by measurement of peak vs. valley ratios,
20 which has also been used to measure degree of
preassociation,^{14,15} was carried out. For a single
pyrene alone a peak:valley ratio of 2.7 was measured,
whereas for the multilabeled DNAs the values
decreased: for 2,3,4,5 and 7 labels the values were
25 1.74, 1.29, 1.54, 1.38, and 4.0 respectively. Thus,
the most intense excimers also may be the most highly
preassociated cases by this measure.

Effect of neighboring bases. The effect of a cytosine
30 versus an adenosine neighboring base was tested

1 (sequences HT3B vs. HT3C). The oligomer where the
neighboring base is adenosine was twice as intensely
fluorescent as the corresponding oligomer with pyrenes
adjacent to a cytosine (Table 3). It is not yet known
5 whether this apparent quenching is due to a lowering
of absorptivity or of quantum yield. In any case,
this apparent quenching appears to hold for pyrene
excimers as well as monomer emission.

10 **Hybridization effects.** To test whether addition of
pyrene labels affects the ability of the DNA to bind
its complement, thermal denaturation studies of the 3'
and 5' series with the complementary 18mer DNA. The
results are shown in Table 4. In general, there is
15 little effect on melting temperatures (T_m) as compared
to the unlabeled sequence (HTO). Addition of pyrenes
to the 3' end is somewhat stabilizing until six or
seven labels are present, in which case the labels are
slightly destabilizing. Addition of pyrene labels to
20 the 5' end is somewhat more stabilizing than the 3'
end, presumably because stacking is more favorable at
the 5' end of a DNA helix.³⁵

Also examined was the effect of hybridization on
fluorescence emission intensity (Table 3). In general
there is very little effect, with intensity decreasing
25 or increasing to a small degree on binding. One
notable exception is the sequence containing a single
pyrene at the 3' end (HT1): On binding the tel3
sequence there is a 20-fold drop in fluorescence
intensity. Examination of the complementary strand
30 shows that thymine is present at the end where the

1 label is situated on the probe strand; it seems likely
that the binding of the complement causes pyrene to
stack not only on adenine but also on its partner
(thymine), which causes strong quenching of the
pyrene.

5 Results with the alpha- and beta-isomeric pyrene
free nucleosides show clearly that, as expected, they
possess essentially the same fluorescence properties
in methanol. However, in a number of different
10 sequences the beta-pyrene exhibits much weaker
fluorescence emission than does the alpha-anomeric
species. This is true both with single substitution
in DNA and with multiply-substituted excimer-emitting
species (Fig. 20). Separate studies have established
15 that the beta-substituted pyrene anomer stacks quick
strongly with natural beta-oriented DNA strands, while
the alpha-substituted case stacks less strongly.³⁵
Similarly, placement of a single pyrene nucleoside
into DNA results in ca. 17-fold lower emission
20 intensity than that for free pyrenemethylamine·HCl in
aqueous buffer, also consistent with quenching by
covalent attachment to the DNA. Also, an adjacent
adenine is less strongly quenching than cytosine or
thymine, which has also been observed with other
pyrene labels.^{30b}

25 In two different sequence contexts a surprising
effect on excimer emission was observed as the number
of contiguous pyrenes increases. First, excimer
emission intensity tends generally to increase up to
five labels and then decreases beyond this number.
30 Second, and even more striking, is that there appears

to be an even/odd effect on fluorescence properties.
1 Three and five labels gives strong excimer intensity,
while, surprisingly, four or six labels gives much
lower intensity (by ca. 6-20 fold). Although
5 intensity drops off strongly beyond five labels, the
even/odd effect extends at least through seven labels,
which also is somewhat more intense than six labels.

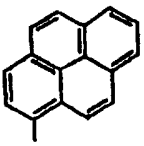
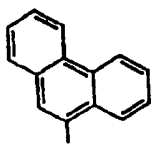
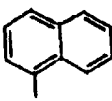
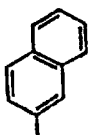
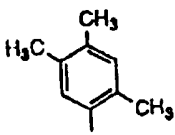
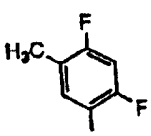
Studies of the absorption and excitation spectra
for the series containing one through seven contiguous
10 alpha pyrene yields some information on the origins of
this effect. It is clear that the extinction
coefficient of a given pyrene-tagged molecule (viewing
a group of pyrenes as a single molecular entity)
varies with the emission intensity. Thus, the
15 even/odd effects are seen on ground state, and it may
not be necessary to invoke special excited-state
interactions to explain the effect. Rather, it
appears that the ground-state structure or electronic
interaction of the multiple pyrene groups may be the
20 primary source of the effects.

25

30

35

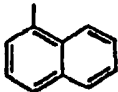
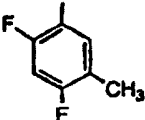
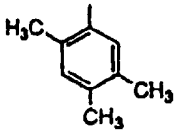
1 **TABLE 1**
H1'-H2' COUPLING CONSTANTS AND PROTON NOE DATA
FOR BETA ISOMERS OF ARYL NUCLEOSIDES 1-6 IN CD₃OD.

Aryl substituent	J values, ^a H1'-H2'	Irradiation at:		
		H1'	H2' α	H2' β
NOE Observed:				
5 	3.6, 10.8 Hz	H1' -%	8%	0%
		H2' α 7	--	--
		H3' 0	0	0
		H4' 6	--	--
10 	3.5, 10.5 Hz	H1' --%	8%	0%
		H2' α 8	--	--
		H3' 0	0	12
		H4' 6	--	--
15 	3.2, 10.7 Hz	H1' --%	8%	0%
		H2' α 7	--	--
		H3' 0	0	5
		H4' 6	--	--
20 	2.6, 10.9 Hz	H1' --%	10%	0%
		H2' α 7	--	--
		H3' 0	0	12
		H4' 6	--	--
25 	2.5, 10.8 Hz	H1' --%	8%	--
		H2' α 9	--	--
		H3' 0	0	--
		H4' 6	--	--
30 	2.3, 10.2 Hz	H1' --%	--	--
		H2' α 9	--	--
		H3' 0	--	--
		H4' 6	--	--

30 ^aCoupling constants are for bis-toluoyl ester derivatives of 1-6 in CDCl₃.

1
 5
 10
 15
 20
 25
 30
 35

TABLE 2
PROTON H1'-H2' COUPLING CONSTANTS AND NOE
DATA FOR ALPHA ISOMERS OF ARYL NUCLEOSIDES
(COMPOUNDS 3A, 5A, AND 6A) IN CD₃OD.

Aryl substituent	J values, ^a H1'-H2'	NOE data: Irradiation at:				
		H1'	H2' α	H2' β	H3'	
 (alpha isomer)	8.0, 6.0 Hz	NOE Observed:				
		H1'	-%	0%	8%	0%
		H2' α	0	--	--	0
		H2' β	8	--	--	6
 (alpha isomer)	7.6, 7.6 Hz	H1'	--%	0%	9%	0%
		H2' α	0	--	--	0
		H2' β	8	--	--	8
		H3'	--	0	8	--
 (alpha isomer)	6.6, 6.6 Hz	H1'	--%	2%	12%	--%
		H2' α	0	--	--	--
		H2' β	6	--	--	--
		H3'	2	4	12	--

^aCoupling constants are for toluoyl ester derivatives in CDCl₃.

1

Table 3 Fluorescence data for pyrene-labeled oligodeoxynucleotides in aqueous buffer.^a Sequences are given in Fig. 2.

sequence	# pyrenes, location		absorption		emission	
			λ_{\max}	ϵ_{348}	λ_{\max}	Intensity (rel) ^b
<u>unhybridized</u>						
HT1	1	3' end	331,348	3.2E4	396	1
HT2	2	"	331,348	5.9E4	489	3.2
HT3	3	"	333,348	1.8E5	489	18
HT4	4	"	333,348	1.5E5	489	3.6
HT5	5	"	335,351	5.3E5	489	31
HT6	6	"	333,349	1.8E3	487	1.5
HT3A3	6	"	--	--	484	27
HT7	7	"	333,350	1.6E4	483	3.4
HT3B	3	5' end	--	--	488	7.5
HT4B	4	"	--	--	489	2.6
HT5B	5	"	--	--	481	17
HT6B	6	"	--	--	475	3.0
HT3C	3	"	--	--	486	3.9
<u>hybridized</u>						
HT1 • tel3	1	3' end	--	--	384	0.05
HT2 • tel3	2	"	--	--	490	5.3
HT3 • tel3	3	"	--	--	487	14
HT4 • tel3	4	"	--	--	490	4.4
HT5 • tel3	5	"	--	--	489	24
HT6 • tel3	6	"	--	--	489	1.2
HT7 • tel3	7	"	--	--	482	1.7

^aConditions: 10 mM PIPES (pH 7.0), 10 mM MgCl₂, 100 mM NaCl, 0.1 μ M in each strand.^bIntegrated emission peak area (425-600nm), relative to that of HT1 (350-425nm).

30

35

1

5

Table 4 Thermal denaturation data (T_m , °C) for pyrene-labeled oligonucleotides hybridized to a short complementary sequence (tel3 or tel3B).^a

sequence	# pyrenes	location	T_m (°C)
HT0	0	-	63.4
HT1	1	3' end	65.4
HT2	2	"	65.9
HT3	3	"	66.1
HT4	4	"	66.4
HT5	5	"	65.8
HT6	6	"	61.1
HT7	7	"	60.0
HT3B	3	5' end	68.3
HT4B	4	"	66.5

15

20

25

30

35

^aConditions: 10 mM PIPES (pH 7.0), 10 mM MgCl₂, 100 mM NaCl, 2 μM in each strand.

REFERENCES:

- 1
1. Santa Lucia, J.; Kierzek, R.; Tumer, D. H. *Science* 1992, 256, 217.
- 5
2. (a) Smith, S. A.; Rajur, S. B.; McLaughlin, L. W., *Vature Struct. Biol.* 1994, 1, 198.
(b) Lesser, D.R.; Kurpiewski, M. R.; Jen-Jacobson, L. *Science* 1990, 250, 776.
- 10
3. (a) Kornberg, A.; Baker, T. A. "DNA Replication", 2nd ed., W. H. Freeman: New York, 1992. (b) Echols, H.; Goodman, M.F. *Ann. Rev. Bioch.* 1991, 60, 477.
(c) Strazewski, P.; Tamm C. *Angew. Chem. Int. Ed. Engl.* 1990, 29, 36.
- 15
4. Kempe, T.; Sundquist, W.I.; Chow, F.; Ho, S.L. *Nucleic Acids Res.* 1985, 13, 45.
- 20
5. Zischler, H.; Nanda, I.; Schafer, R; Schmid, M.; Epplen, J.T. *Hum. Genet.* 1989, 82, 227.
- 25
6. (a) Weygand-Durasevic, I.; Susic, S. *Biochim. Biophys. Acta* 1990, 1048, 38. (b) Hustedt, E. J.; Spaltenstein, A.; Kirchner, J.J.; Hopkins, P.B.; Robinson. B.H. *Biochemistry* 1993, 32, 1774.
- 30
- 35

- 1 7. Dreyer, G.B.; Dervan, P.B. *Proc. Natl. Acad. Sci. USA* 1985, 82, 968.
- 5 8. Beaucage, S. L.; Iyer, R.P. *Tetrahedron* 1993, 49, 1925.
- 10 9. (a) Ansorge, W.; Sproat, B.S.; Stegemann, J.; Schwager, C.J.; *Biochem. Biophys. Methods* 1986, 13, 315. (b) Karger, A.E.; Harris, J. M.; Gesteland, R.F. *Nucleic Acids Res.* 1991, 19, 4955.
- 15 10. (a) Ward, D.C.; Reich, E.; Stryer, L.J. *Biol. Chem.* 1969, 244, 1228. (b) Bloom, L.B.; Otto, M.R.; Beechem, J.M.; Goodman, M.F. *Biochemistry* 1993, 32, 11247.
- 20 11. (a) Secrist, J.A.; Barrio, J.R.; Leonard, N.J. *Science* 1972, 175, 646. (b) Toulme, J.J.; Helene, C. *Biochim Biophys. Acta* 1980, 606, 95.
- 25 12. Schweitzer, B.A.; Kool, E.T. *J. Org. Chem.* 1994, 59, 7238.
- 30 13. (a) Chaudhuri, N.C.; Kool, E.T. *Tetrahedron Lett.* 1995, 1795. (b) Chandhuri, N.C.; Kool, E.T. *ibid*, 4910.
- 35 14. Schweitzer, B.A.; Kool, E.T. *J. Am. Chem. Soc.* 1995, 117, 1863.

- 1
15. Hunter, C.A. *Angew. Chem., Int. Ed. Engl.* 1993,32, 1584.
- 5
16. (a) Matsumoto, T.; Katsuki, M.; Suzuki, K. *Tetrahedron Lett.* 1988, 29.6935. (b) Ohrui, H.; Kuzubara, H.; Emoto, S. *Agr. Biol Chem. (Tokyo)* 1972,36, 1651-1653.
- 10
17. Klein, R.S.; Kotick, M.P.;Watanabe, K.A.; Fox J.J. *J. Org. Chem.* 1971,36.4113.
- 15
18. Millican, T.A.; Mock, G.A.; Chauncey, M.A.; Patel T.P.; Eaton, M.A.W., Gunning, J.; Cutbush, S.D.; Neidle, S.; Mann, J. *Nucleic Acids Res.* 1984, 12, 7435.
19. Hoffer, M. *Chem. Ber.* 1960, 93, 2777.
- 20
20. Davies, D.B. *Prog. NMR Spectrosc.* 1978, 12,135.
- 25
21. (a) Schulman, S.G. *Molecular Luminescence Spectroscopy*, Wiley: New york, 1990, 1-27. (b) Slavik J. *Fluorescent Probes in Cellular and Molecular Biology*, CRC Press: Boca Raton, 1990, 1-36.
- 30
24. Cleve, G.; Hoyer, G.; Schulz, G.; Vorbruggen, H. *Chem. Ber.* 1973, 106, 3062.

- 1 25. Robins, M.J.; Robins, R.K. *J. Am. Chem. Soc.* 1965, 87, 4934.
- 5 26. (a) Srivastava, P.C.; Robins, P.K.; Takusagawa, F.; Berman, H.M. *J. Het. Chem.* 1981, 18, 1659. (b) Hacksell, U.; Cheng, J. C-Y.; Daves, G.D., Jr. *Nucleosides & Nucleotides* 1986, 5, 287.
- 10 28. Sugimoto, N.; Kierzek, R.; Tumer, D.H. *Biochemistry* 1987, 26, 4554.
29. Senior, M.; Jones, R. A.; Breslauer, K.J. *Biochemistry* 1988, 27, 3879.
- 15 30. (a) Telser, J.; Cruickshank, K.A.; Morrison, L.E.; Netzel, T.L.; Chan, C. *J. Am. Chem. Soc.* 1989, 111, 7226. (b) Telser J.; Cruickshank, K.A.; Morrison, L.F.; Netzel, T.L. *J. Am. Chem. Soc.* 1989, 111, 20 6966. (c) Lee, H.; Hinz, M.; Stezowski, J.J.; Harvey, R.G. *Tetrahedron Lett.* 1990, 31, 6773. (d) Yamana, K.; Gokota, T.; Ozaki, H.; Nakano, H.; Sangen, O.; Shimidzu, T. *Nucleosides Nuclotides* 1992, 25 11, 383. (e) Prokhorenko, I.A.; Petrov, A.A.; Gontarev, S.V.; Berlin, Y.A. *BioMed. Chem. Lett.* 1995, 5, 2081. (f) Li, Y.; Bevilacqua P.C.; Mathews, D.; Turner, D.H. *Biochemistry* 1995, 34, 14394. (g) Tong, G.;
- 30
- 35

- 1 Lawlor, J.M.; Tregear, G.W.; Haralambidis,
J. *J Am. Chem. Soc.* 1995, 117, 12151.
31. Moran, S.; Ren, R.X.-F.; Sheils, C.J.;
5 Kool, E.T. *Nucleic Acids Res.* 1996, 24,
2044.
32. Ren, R.X.-F.; Chaudhuri, N.C.; Paris, P.L.;
Rummey IV, S.; Kool, E.T. *J. Am. Chem. Soc.*
10 1996, 118, 7671.
33. Borer, P.N. in *Handbook of Biochemistry and
Molecular Biology*, G.D. Fismann, ed., CRC
Press: Cleveland, 1975, p. 589.
- 15 34. Moyzis, R.K.; Buckingham, J.M.; Cram, L.S.;
Dani, M.; Deaven, L.L.; Jones, M.D.; Meyne,
J.; Ratliff, R.L.; Wu, J.-R. *Proc. Natl.
Acad. Sci. USA* 1988, 85, 6622.
- 20 35. Guckian, G.; Schweitzer, B.A.; Ren, R. X.-
F.; Sheils, C.J.; Paris, P.L.; Tahmassebi,
D.C.; Kool, E.T. *J. Am. Chem. Soc.* 1996,
118, 8182.

25

30

35

WHAT IS CLAIMED IS:

1

1. A nucleoside derivative comprising a pyrene, anthracene or phenanthrene joined to the C-1 atom of a sugar moiety through a carbon-carbon bond in either an α or β configuration wherein said sugar moiety is ribose or deoxyribose.

5

2. α -9-phenanthrenyl deoxynucleoside.

10

3. α -1-pyrenyl deoxynucleoside.

4. β -9-phenanthrenyl deoxynucleoside.

5. β -1-pyrenyl deoxynucleoside.

15

6. A phosphoramidite derivative of the nucleoside derivative of Claim 1.

20

7. The nucleoside derivative of Claim 1 wherein the pyrene, anthracene or phenanthrene is derivatized at an available carbon position with a substituent selected from the group consisting of methoxy, ethoxy, dimethylamino, diethylamino, nitro, methyl, cyano, carboxy, chloro, bromo, iodo and amino.

25

8. A nucleic acid comprising at least one nucleoside derivative according to any of Claims 1-6.

30

35

1 9. A method of synthesizing the nucleoside
derivative of Claim 1 which comprises:

- 5 a. coupling an organocadmium or organozinc
derivative of pyrene, anthracene or phenanthrene to
the C1 position of Hoffer's α -chlorosugar; and
b. removing the protecting groups with a
methanolic base.

10 10. A method of synthesizing a phosphoramidite
derivative of the nucleoside derivative of Claim 1
which comprises:

- 15 a. coupling an organocadmium or organozinc
derivative of pyrene, anthracene or phenanthrene to
the C1 position of Hoffer's α -chlorosugar;
b. removing the protecting groups with a
methanolic base;
c. tritylating the 5'-OH with
dimeoxytritylchloride in the presence of a base; and
d. phosphitylating the 3'-OH with a
phosphitylating agent.

20 11. A method of preparing a fluorescently labeled
nucleic acid which comprises incorporating a
nucleoside derivative of any of Claims 1-6 into an RNA
or DNA molecule under conditions sufficient to
25 incorporate said nucleoside.

30 12. A method of detecting a target nucleic acid
in a sample to be tested comprising contacting the
target nucleic acid with a nucleic acid probe
comprising at least one nucleoside derivative of any

35

1 one of Claims 1-6 for a time and under conditions
sufficient to permit hybridization between said target
and said probe; and detecting said hybridization.

5

10

15

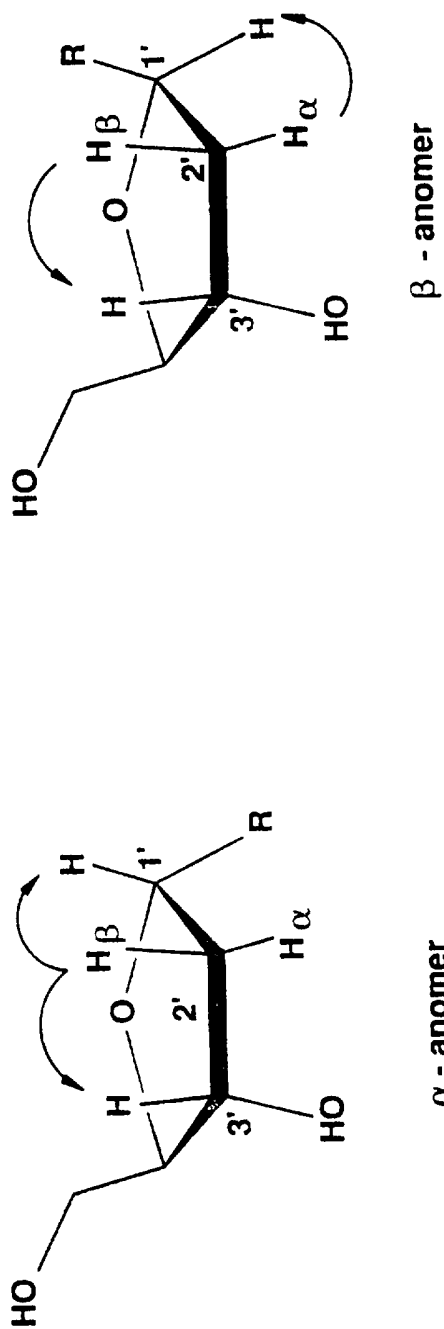
20

25

30

35

FIG.1



Irradiation at :

H α : 0 enhancements
H β : 2 enhancements

H α : 1 enhancements
H β : 1 enhancements

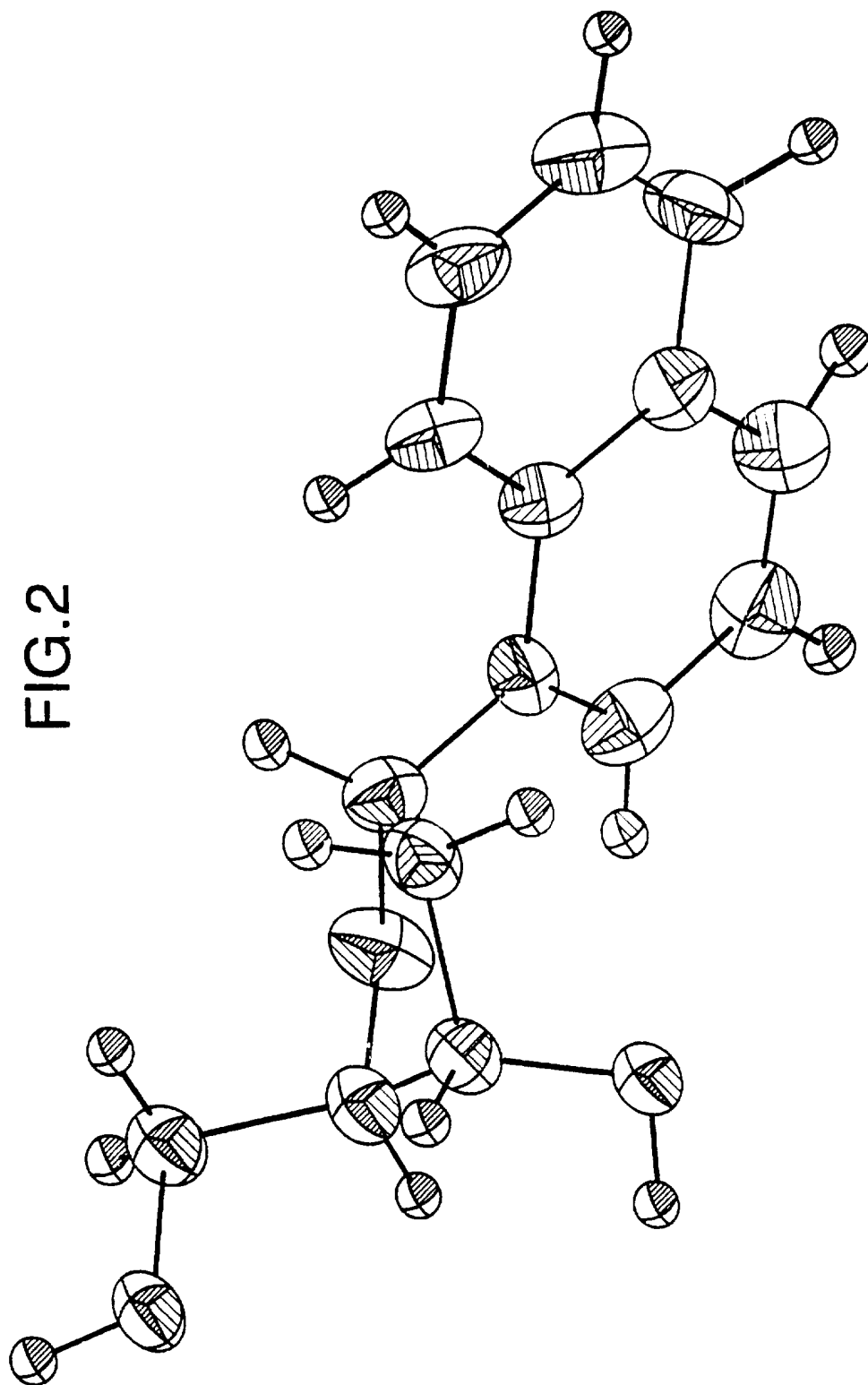
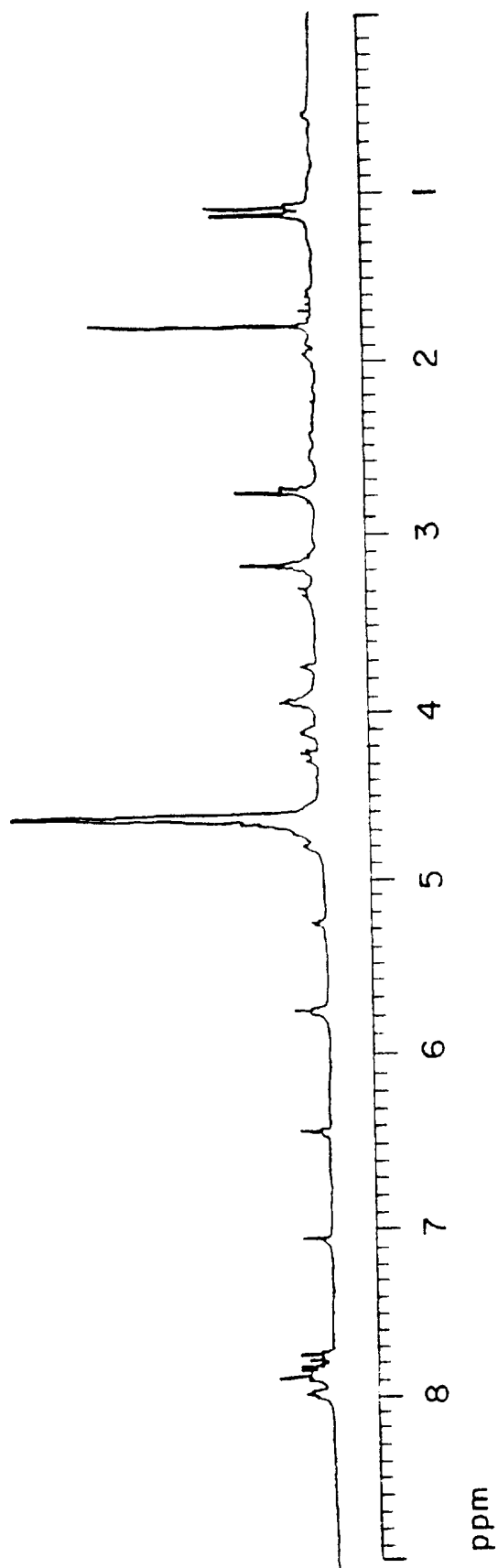


FIG. 2

3/24

FIG. 3A



4/24

FIG. 3B

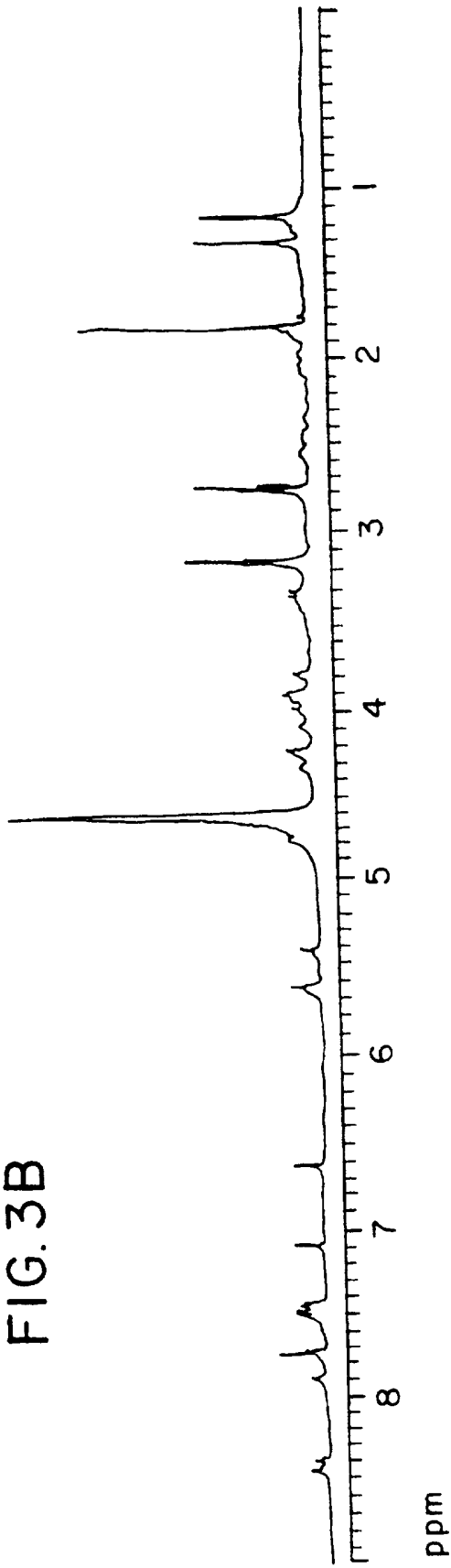


FIG. 3C

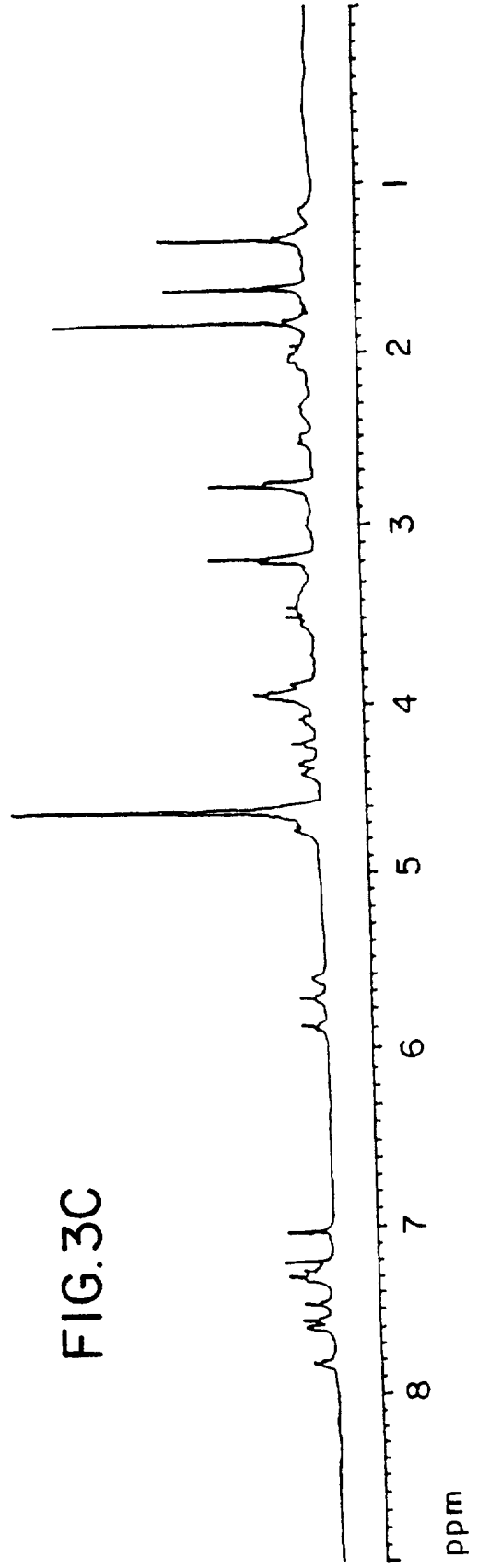


FIG.4A

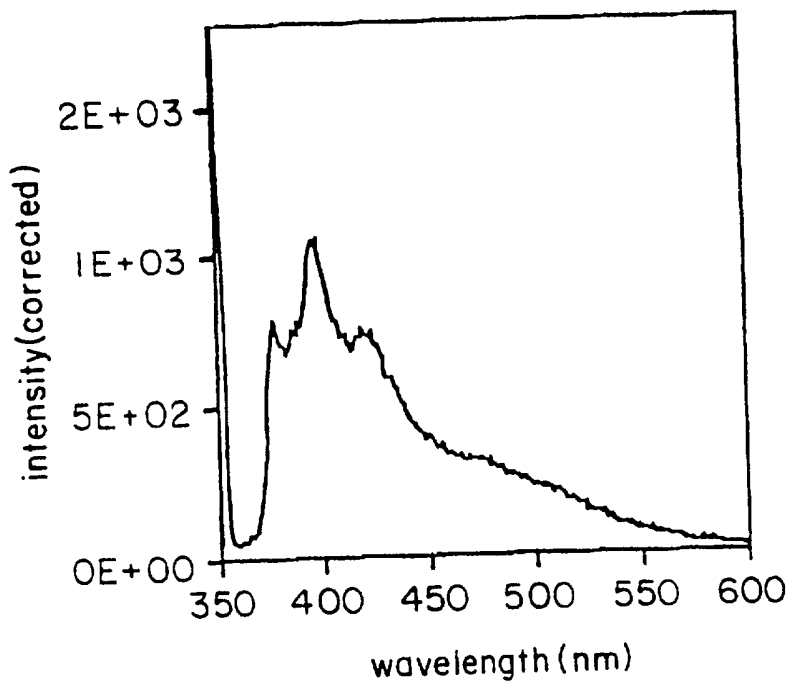


FIG.4B

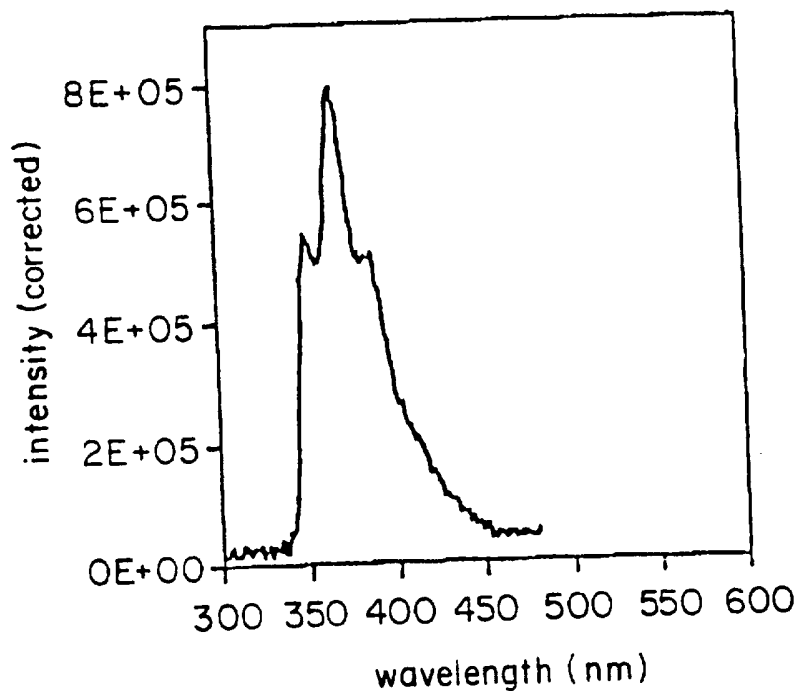
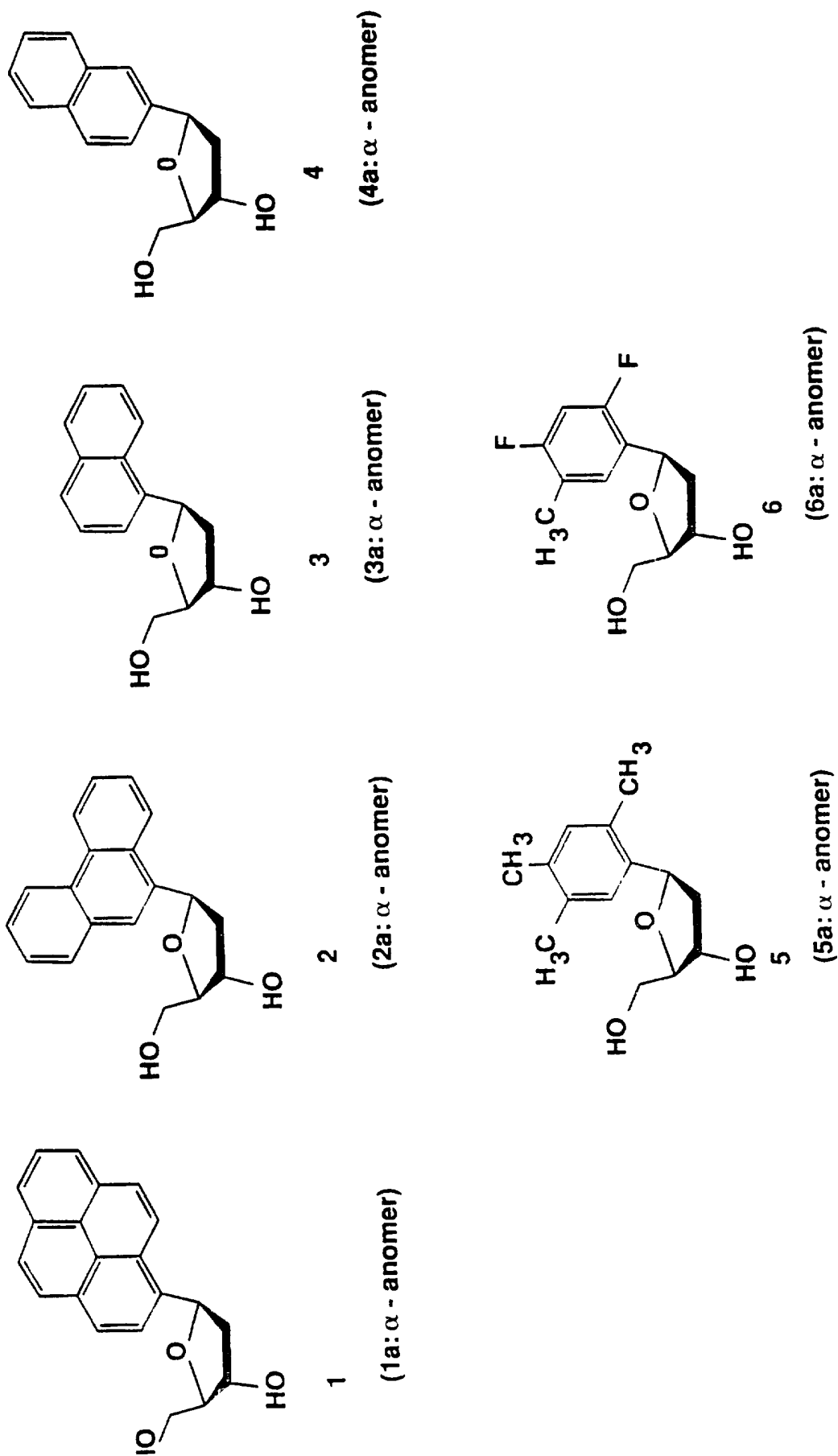


FIG.5



7/24

FIG.6

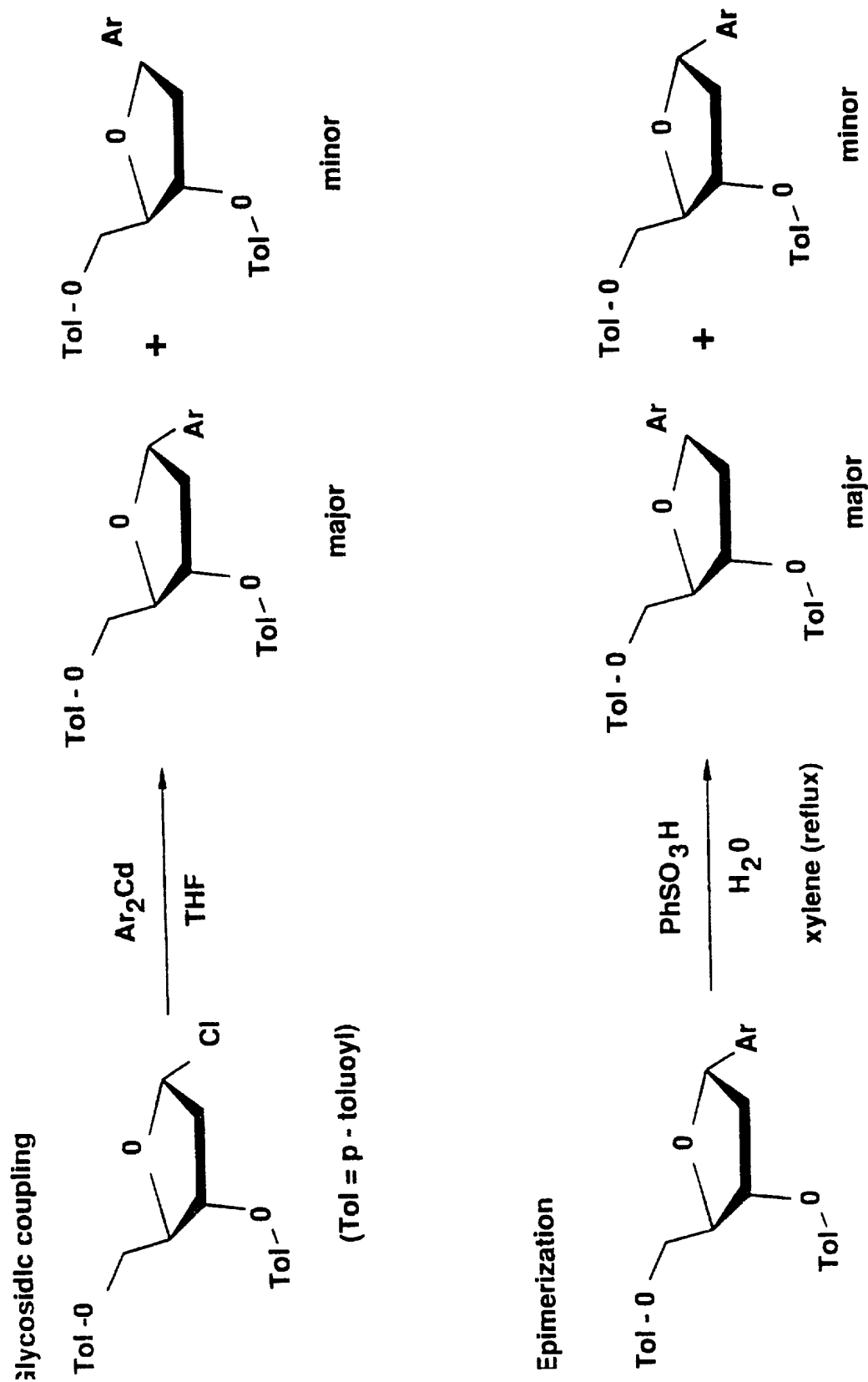
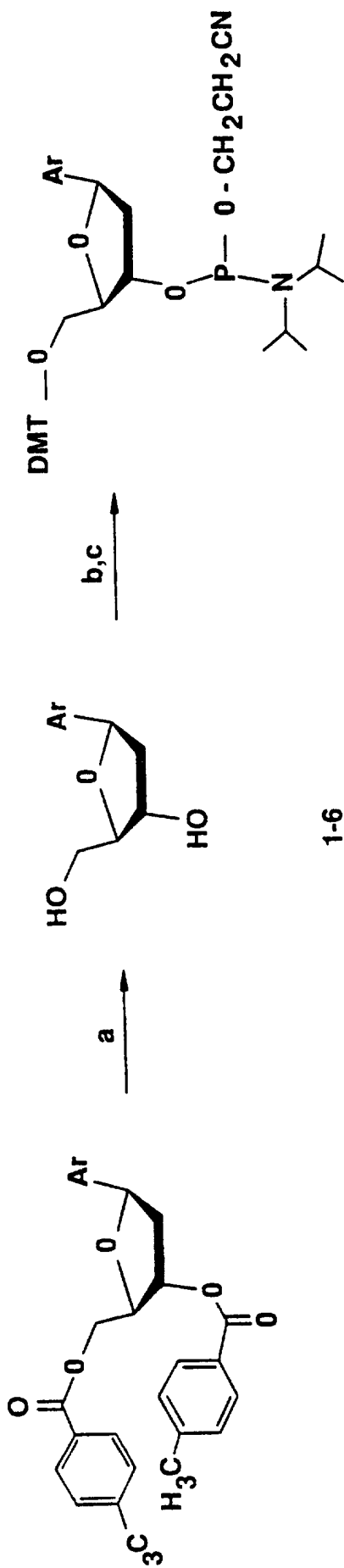


FIG.7

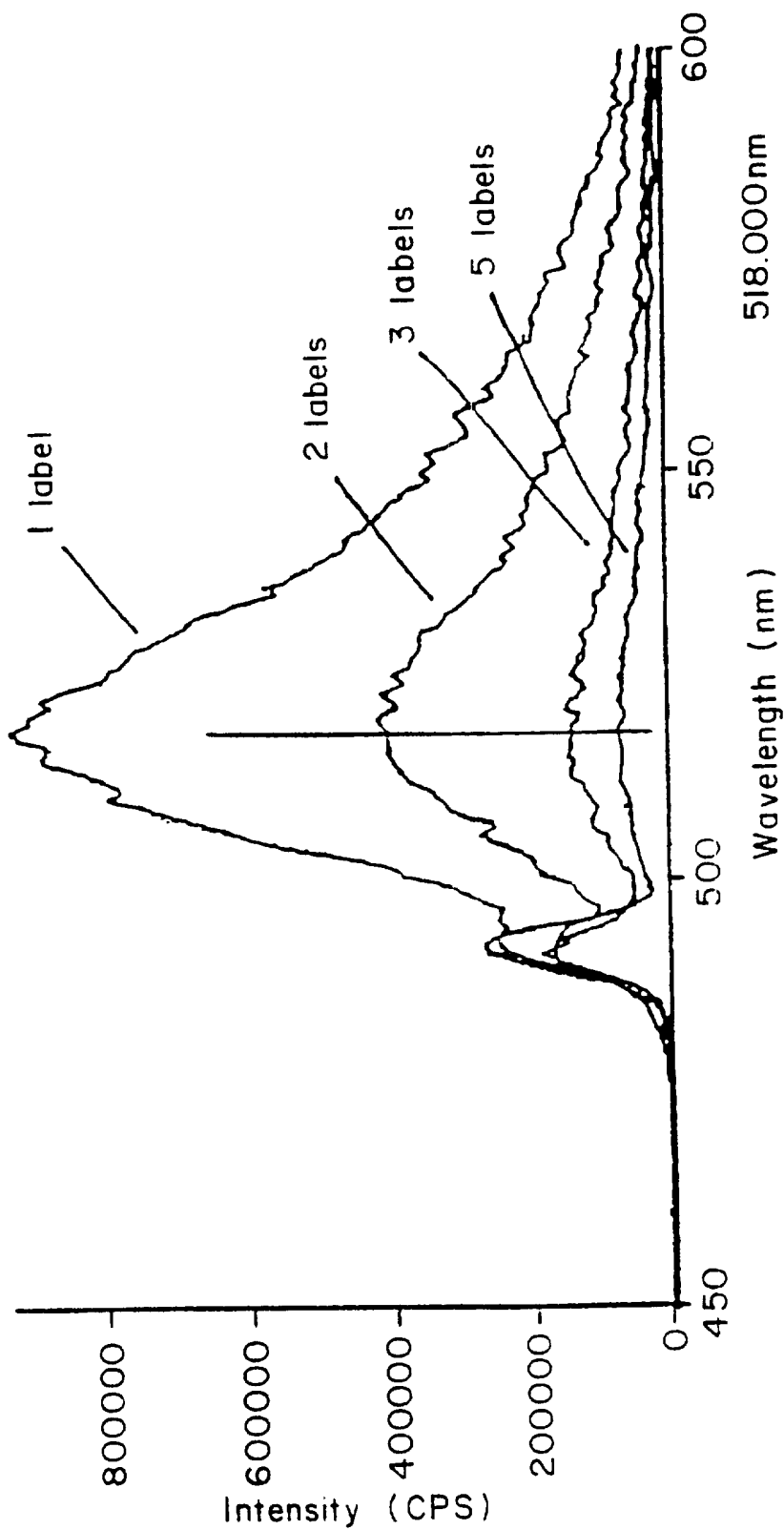


- 1: Ar = 2,4 - difluorotoluy
- 2: Ar = 2,4,5 - trimethylphenyl
- 3: Ar = 1 - naphthyl
- 4: Ar = 2 - naphthyl
- 5: Ar = 2 - phenanthryl
- 6: Ar = 1 - pyrenyl

(a) NaOMe / methanol, 23° C; (b) 4,4' - imethoxytrityl chloride, DMAP , pyridine, CH₂C12,23° C; (c) N,N'-diisopropyl-2 - 0 - cyanoethyl phosphonamidic chloride, DIPEA, CH₂Cl₂, 23° C.

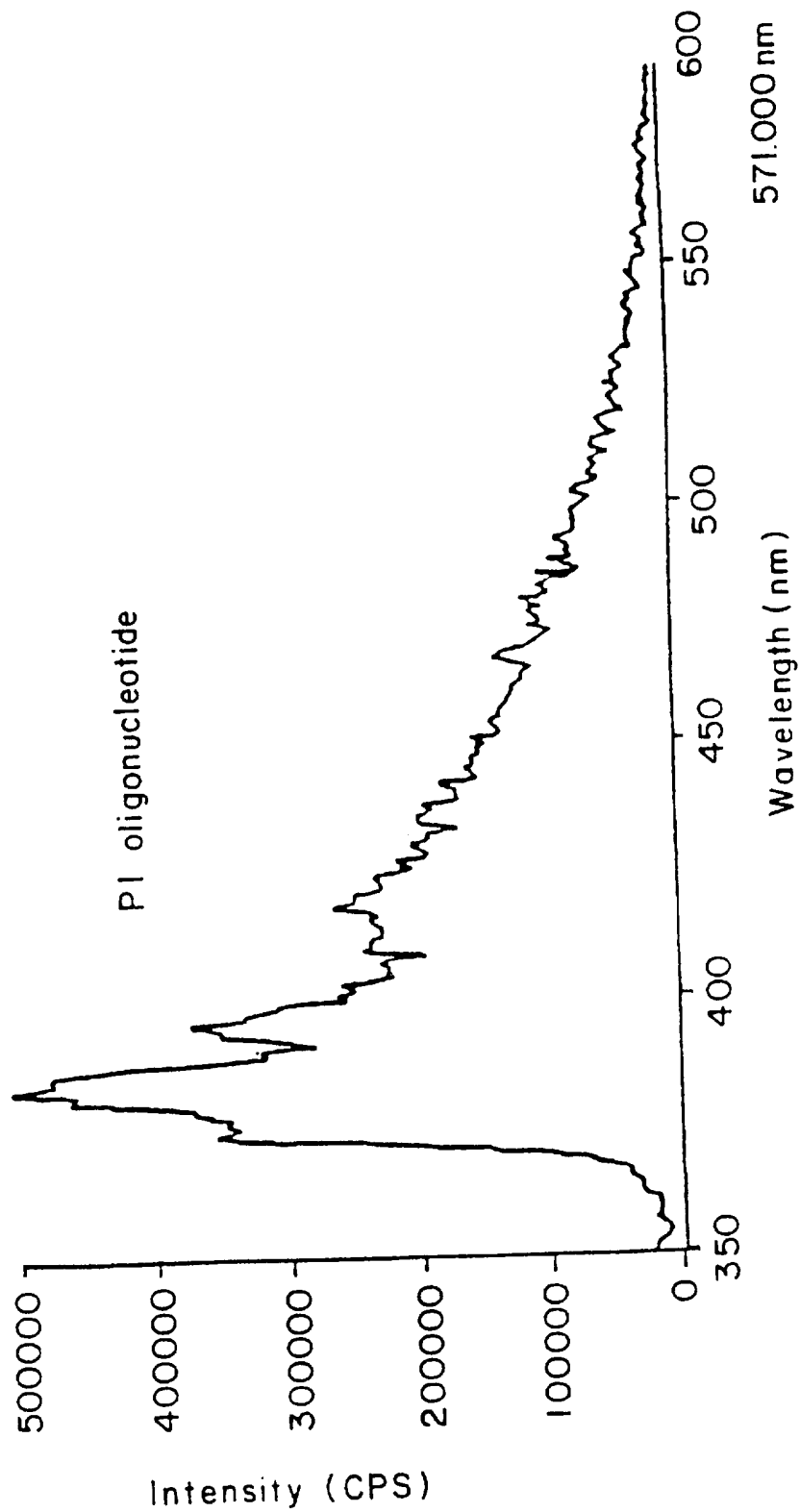
9/24

FIG. 8



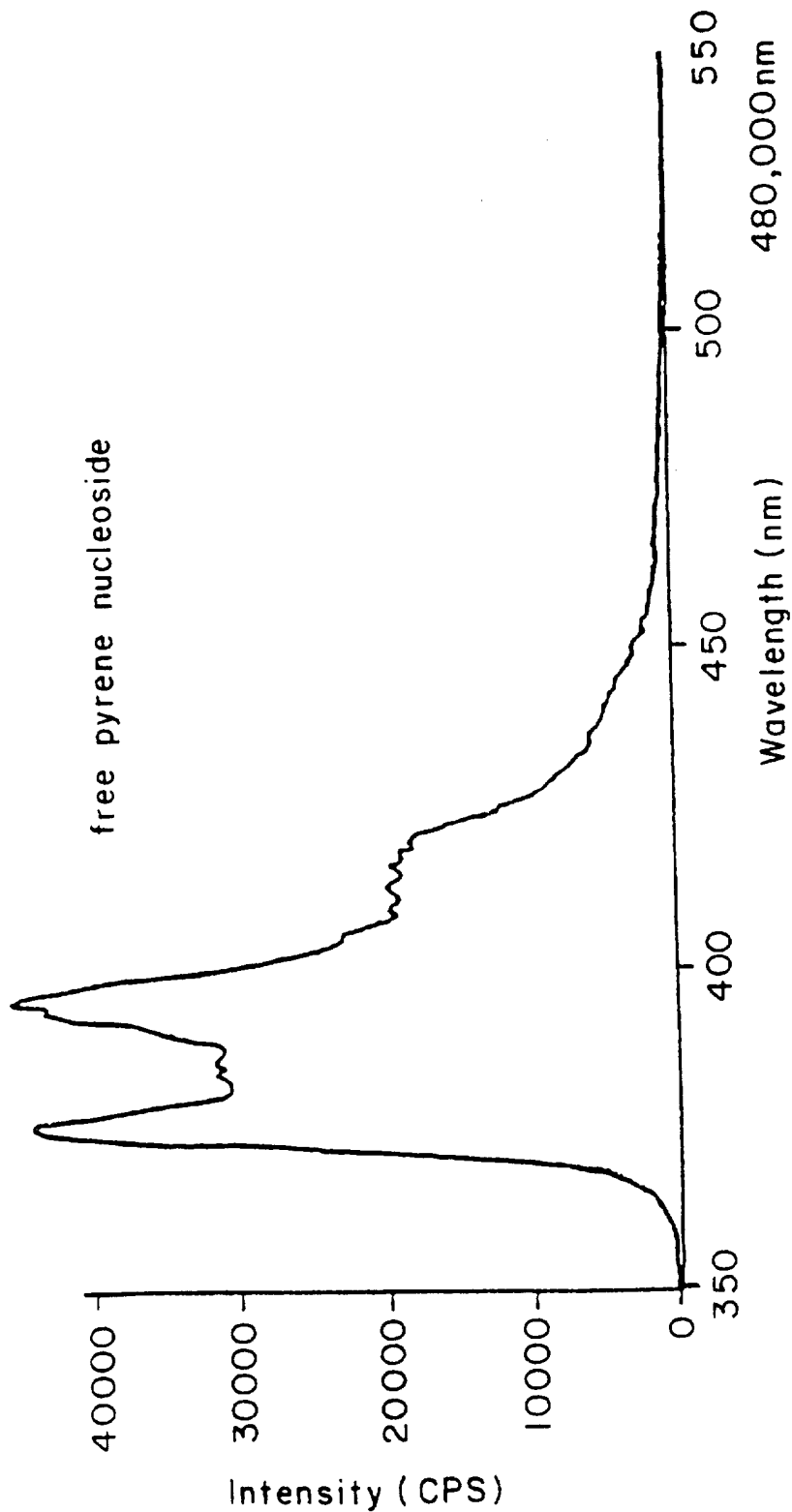
10/24

FIG. 9



11/24

FIG. 10



12/24

FIG. II

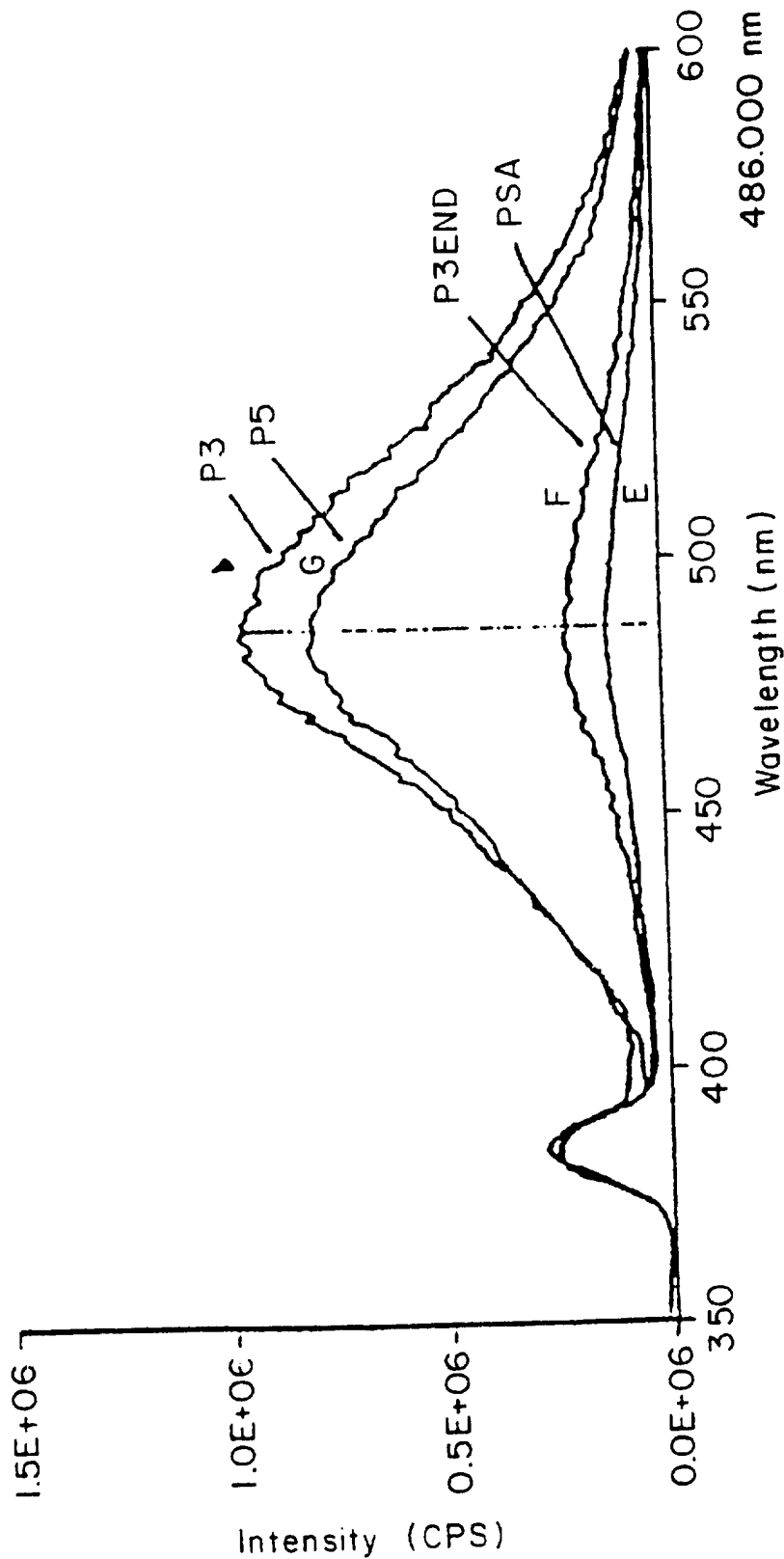
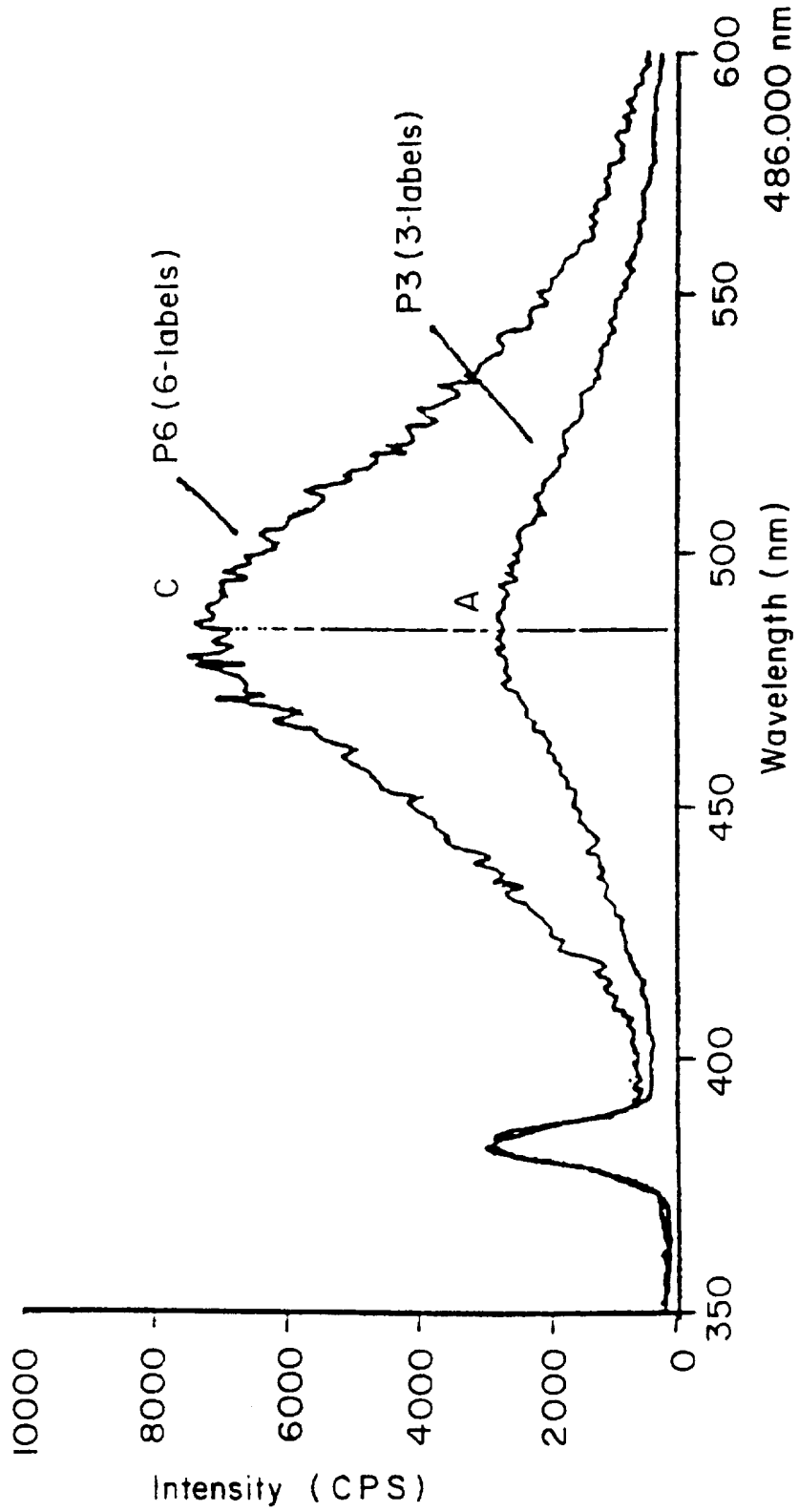


FIG. 12



(P = α -pyrene nucleotide)

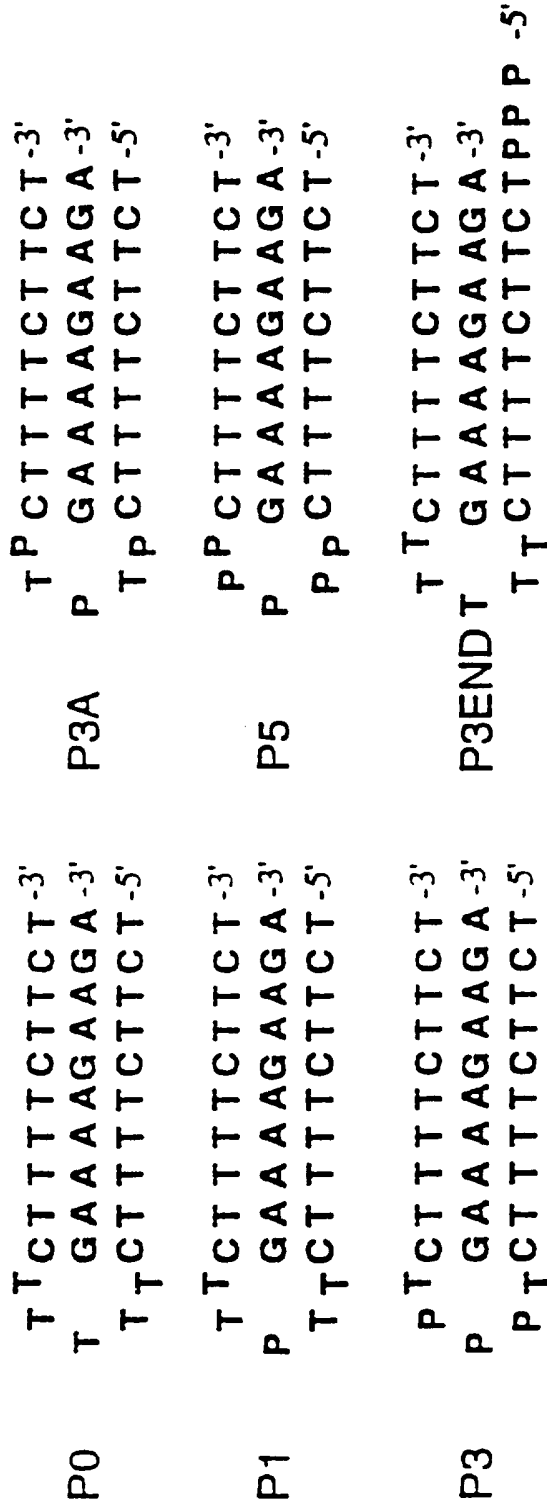


FIG.13

15 / 24

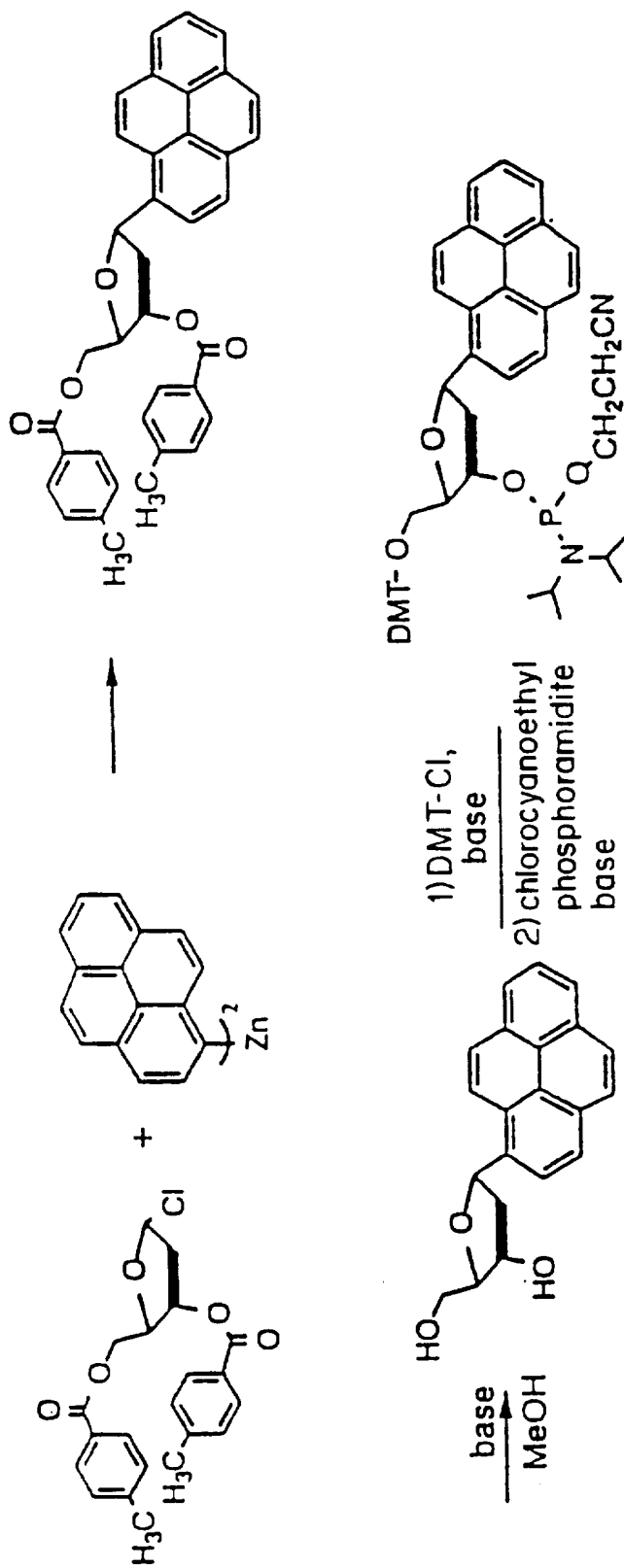


FIG.14

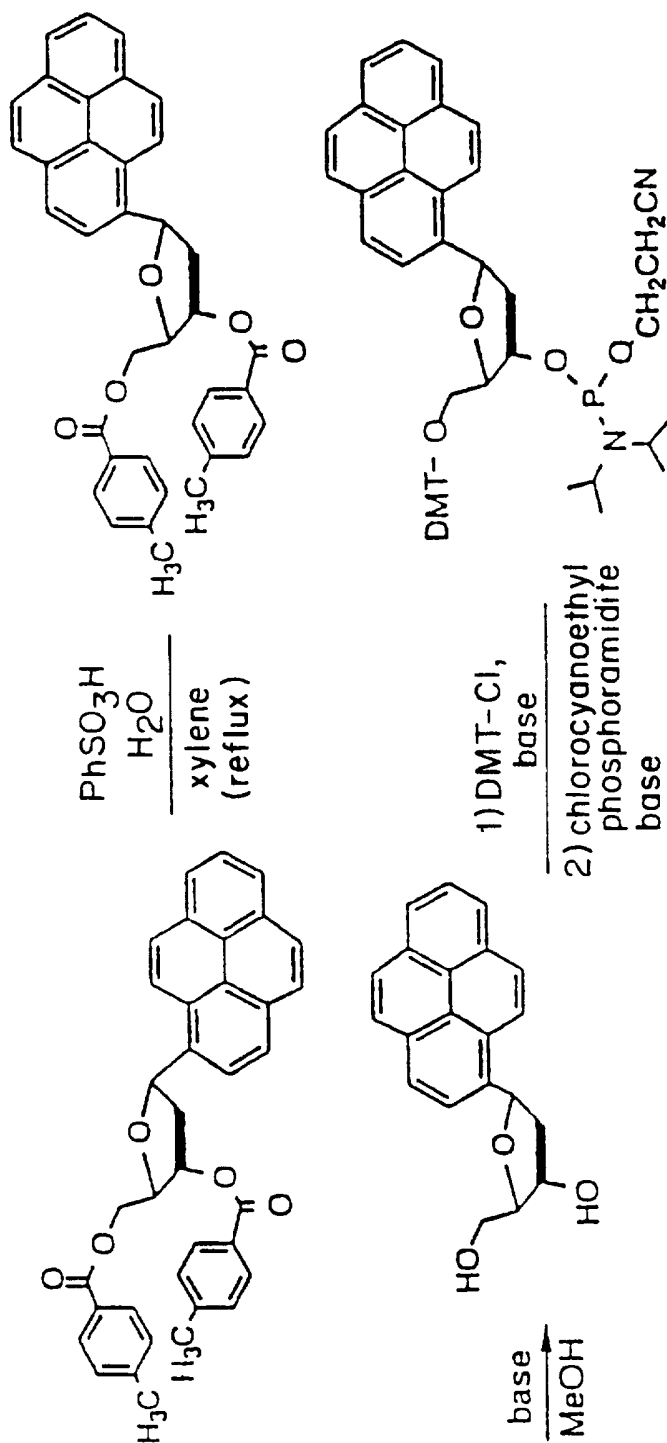


FIG. 15

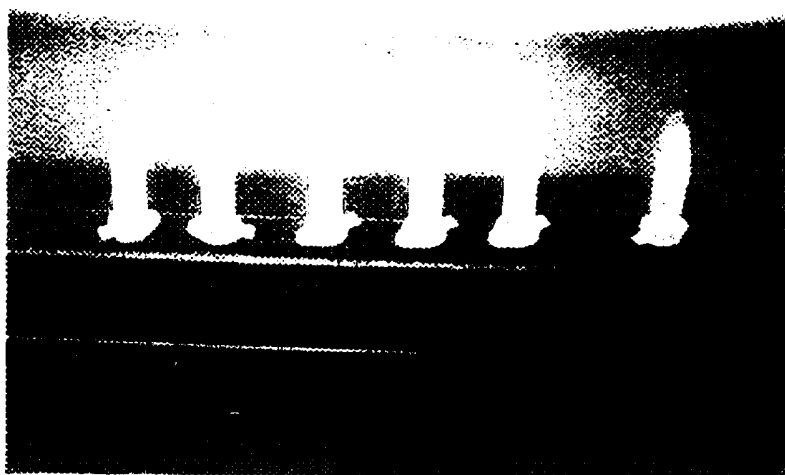


FIG. 16A

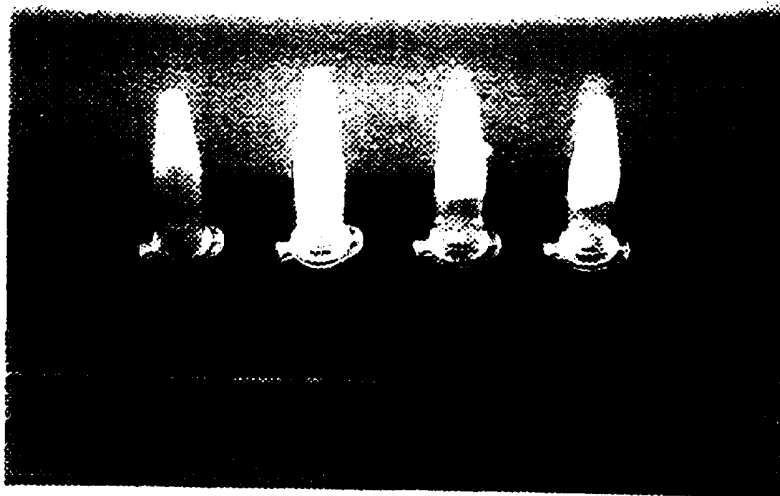


FIG. 16B

18 / 24

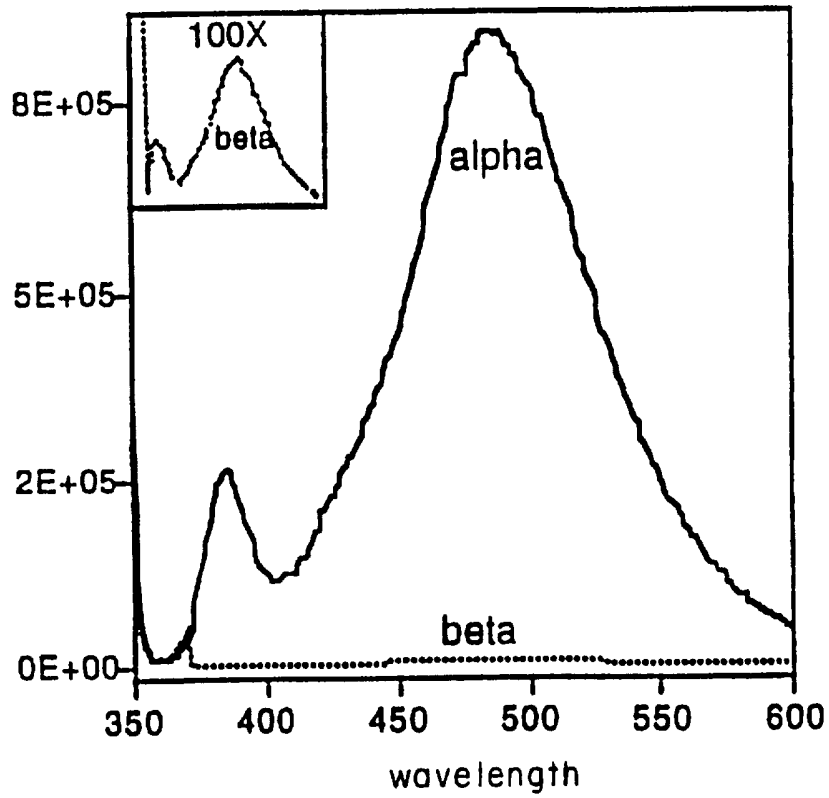


FIG. 17

FIG.18

	<u>α - pyrene sequences</u>	<u>relative intensity</u>
	pyrene nucleoside alone	1.0
P1	T T C T T T T C T T C T -3' P T T C T T T T C T T C T -5'	10
P2	T T C T T T T C T T C T -3' P P T C T T T T C T T C T -5'	11
P3	P T C T T T T C T T C T -3' P P T C T T T T C T T C T -5'	18
P5	P P C T T T T C T T C T -3' P P P C T T T T C T T C T -5'	89
C6	P T C T T T T C T T C T T T T C T P P P T C T T T T C T T C T T C T T T T C T P	37
C10	P P C T T T T C T T C T T C T T T T C P P P P P C T T T T C T T C T T C T T T T C P P	196

20 / 24

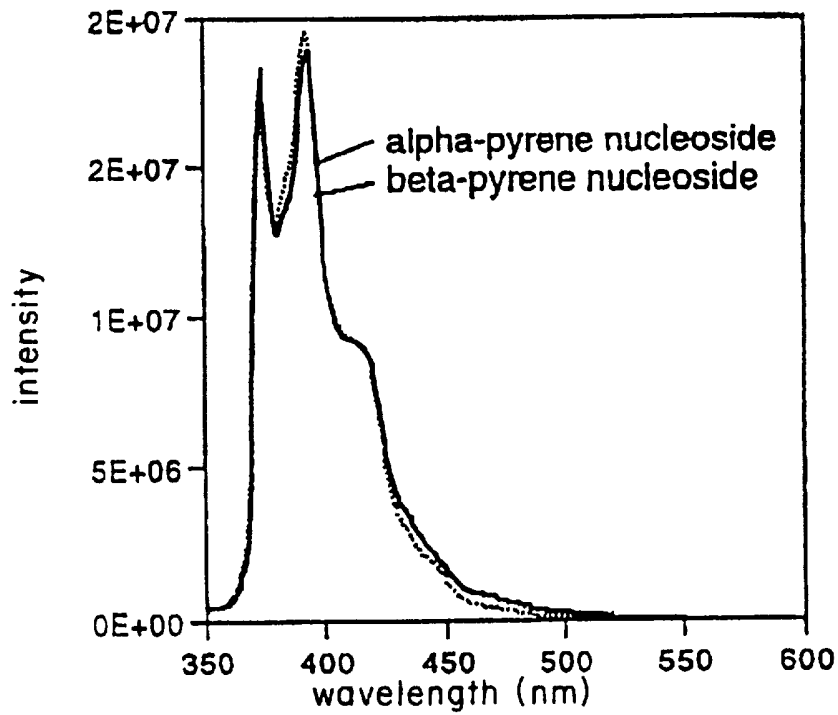


FIG. 19A

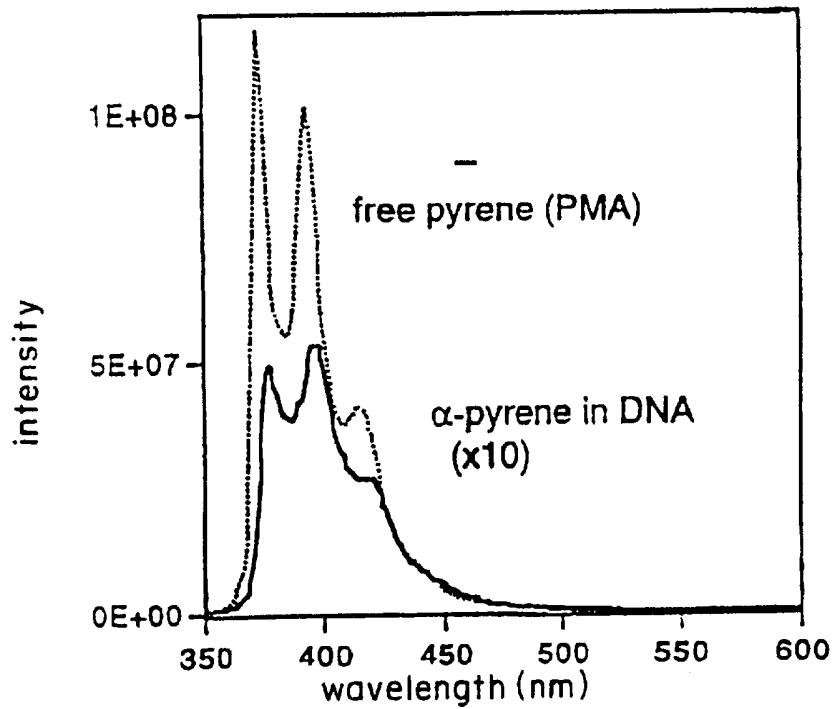


FIG. 19B

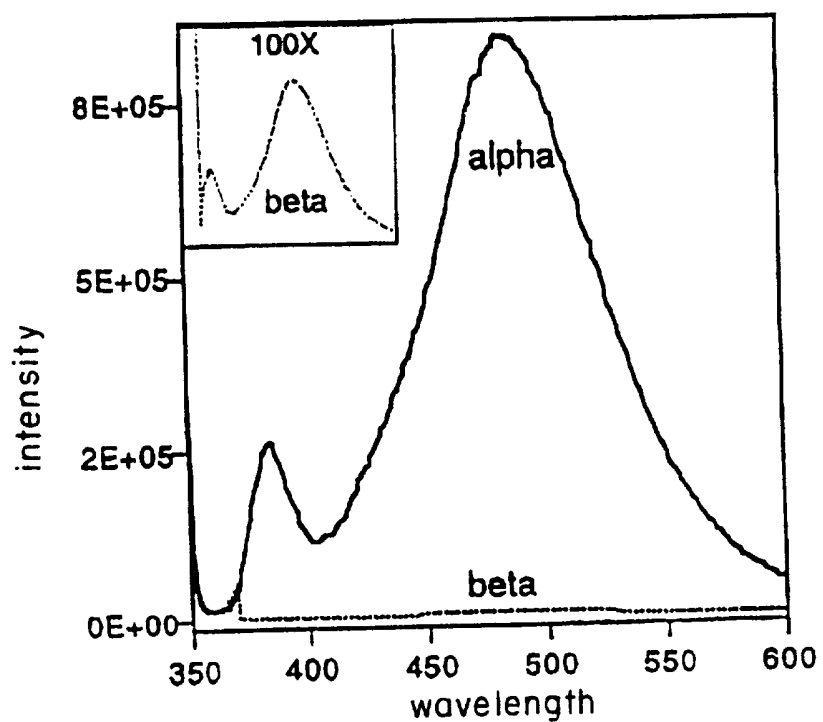


FIG.20

FIG.2IA

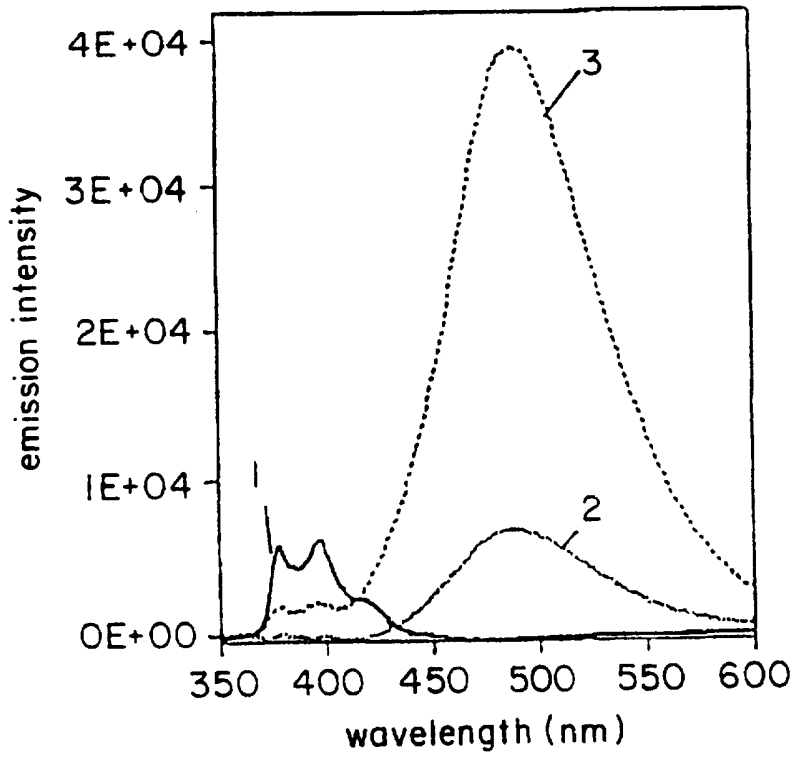


FIG.2IB

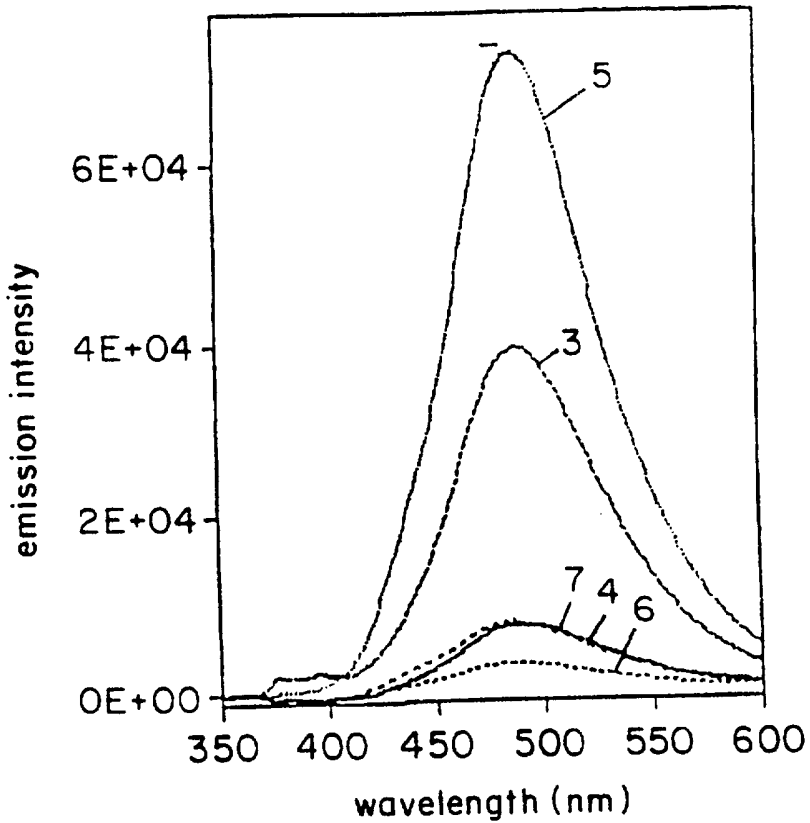
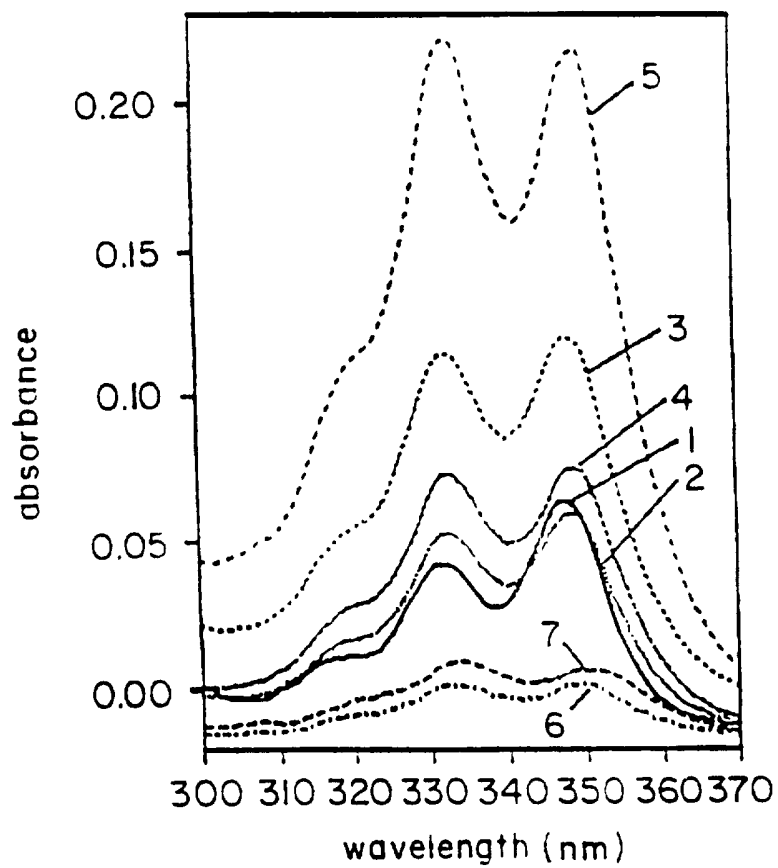


FIG. 22



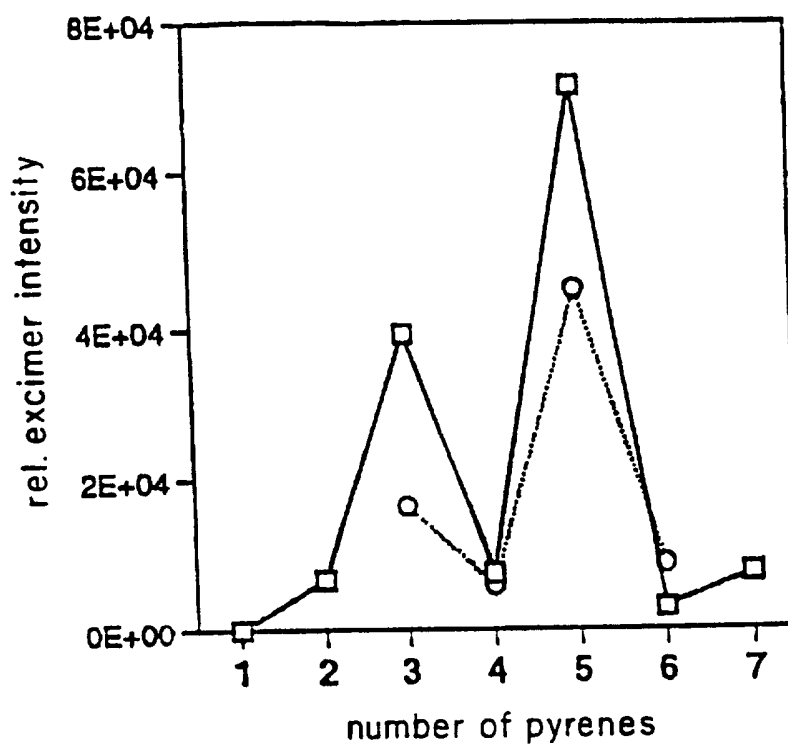


FIG. 23

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/08307

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H19/00 C07D307/06 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H C07D C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	E.T. KOOL ET AL.: "Naphthalene, Phenanthrene and Pyrene as DNA Base Analogues" J. AM. CHEM. SOC., vol. 118, 1996, pages 7671-8, XP002041611 cited in the application see the whole document ---	1-12
P,X	E.T. KOOL ET AL.: "Experimental Measurement of Aromatic Stacking Affinities in the Context of Duplex DNA" J. AM. CHEM. SOC., vol. 118, 1996, pages 8182-3, XP002041612 cited in the application see the whole document ---	1-12
	-/--	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

24 September 1997

Date of mailing of the international search report

14. 10. 97

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+ 31-70) 340-3016

Authorized officer

Bardili, W

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/08307

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>C.R. NEWTON ET AL.: "The Production of PCR Products with 5' Single-Stranded Tails Using Primers That Incorporate Novel Phosphoramidite Intermediates" NUCLEIC ACIDS RES., vol. 21, 1993, pages 1155-62, XP002041613 see page 1158, compound (10). ---</p>	
A	<p>R.L. LETSINGER AND K. YAMANA: "Synthesis and Properties of Oligonucleotides Bearing a Pendant Pyrene Group" NUCLEIC ACIDS RES., vol. 16, 1985, pages 169-172, XP002041614 ---</p>	
A	<p>WO 95 05391 A (CHROMAGEN INC) 23 February 1995 ---</p>	
A	<p>N. BISCHOFBERGER AND M.D. MATTEUCCI: "Synthesis of Novel Polycyclic Nucleoside Analogues" J. AM. CHEM. SOC., vol. 111, 1989, pages 3041-6, XP002041615 -----</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/08307

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9505391 A	23-02-95	CA 2145750 A EP 0669928 A US 5652099 A	23-02-95 06-09-95 29-07-97
