Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 5, pp. 431-436, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00050-X

The Evaluation of 2'- and 6'-substituted Carbocyclic Nucleosides as Building Blocks for Antisense Oligonucleotides

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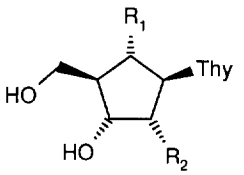
Abstract: Oligodeoxyribonucleotides incorporating 2'- or 6'-alkoxy substituted carbocyclic nucleotide units are shown to bind to complementary RNA with lower affinity than their unmodified parent compounds, while the presence of stretches of contiguous 6'-hydroxy substituted building blocks enhances RNA-binding affinity. 6'-substituted carbocyclic nucleotides are generally found to increase oligonucleotide resistance to enzymatic degradation, the effect being more pronounced for larger substituents.

The specific inhibition of protein expression by oligonucleotide based therapeutic agents has recently emerged as an important alternative to classical drug design strategies.[1] Central to the successful implementation of the underlying concepts is the structural modification of natural oligonucleotides, as the specific interference with protein expression either at the transcriptional (*anti-gene* approach) or translational (*antisense* approach) level *in vivo* not only requires sequence-specific binding of an oligonucleotide drug to the single stranded mRNA or double stranded DNA target, respectively, but also depends on its sufficient metabolic stability under physiological conditions.[1] As a consequence extensive efforts have been devoted in recent years to the design and synthesis of modified oligonucleotides or oligonucleotide analogs with improved nuclease resistance, which still retain (or even exceed) the ability of natural oligonucleotides to bind to complementary nucleic acid targets with high affinity and specificity.[1,2]

Among other approaches modification of the sugar moiety of natural oligodeoxy-ribonucleotides has involved the replacement of the furanose oxygen O-4' by a CH_2 group (*Fig. 1*, $R_1 = R_2 = H$).[3] Oligonucleotides incorporating such *carbocyclic* nucleotide units were found to exhibit favorable RNA-binding properties,[3c,4] but the degree of nuclease resistance conferred by unsubstituted carbocyclic 2-deoxyribonucleotides is unlikely to suffice for biological applications.[3c] However, this problem may be overcome by the further derivatization of the cyclopentane moiety of carbocyclic oligonucleotide building blocks,[5] which could have a significant impact on the nuclease resistance of the corresponding oligonucleotides. In particular, replacement of the furanose oxygen by a carbon atom (designated *C-6'*) offers the unique possibility to introduce additional substituents at this position, which might hamper recognition of so modified oligonucleotides by (single-stranded) DNA degrading enzymes and thus lead to enhanced metabolic stability. At the same time model building indicates that 6'-α-substituents (i. e. trans to the base moiety) can be readily accommodated within a standard A-type DNA/RNA duplex without any structural distortions[6] and, therefore, should not be expected to impede RNA binding affinity, at least not for simple steric reasons.

Figure 1

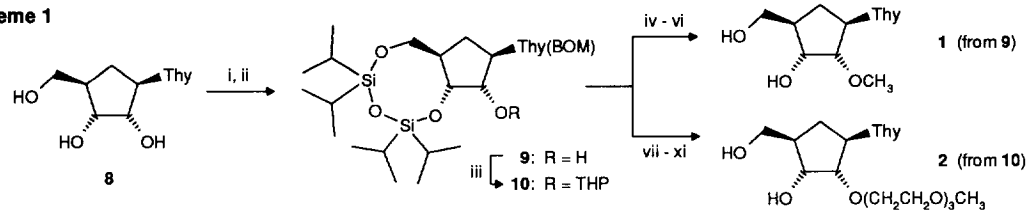


1:	$R_1 = H$,	$R_2 = OCH_3$
2:	$R_1 = H$,	$R_2 = O(CH_2CH_2O)_3CH_3$
3:	$R_1 = OH$,	$R_2 = H$
4:	$R_1 = OCH_3$,	$R_2 = H$
5:	$R_1 = O(CH_2)_4NH_2$,	$R_2 = H$
6:	$R_1 = O(CH_2)_3Ph$,	$R_2 = H$
7:	$R_1 = O(CH_2CH_2O)_2CH_3$,	$R_2 = H$

An alternative approach to improve the nuclease resistance of oligonucleotides incorporating carbocyclic building blocks consists in the attachment of substituents to the 2'-position of the cyclopentane moiety. 2'-Alkoxy[7] as well as 2'-alkyl[8] substituents significantly improve the metabolic stability of natural oligodeoxyribonucleotides, with the former also leading to increased binding affinity for complementary RNA.[9] However, it is important to note that the hybridization properties of 2'-alkoxy substituted oligodeoxyribonucleotides may not be predictive for the case of carbocyclic building blocks, as their increased RNA-binding affinity is generally attributed to a preference of the modified sugar moiety for a 3'-endo conformation due to a favorable *gauche* arrangement of the 2'-hetero-substituent and the furanose oxygen O-4'.[9b,10,11] In this context 2'-alkoxy substituted carbocyclic nucleotide building blocks should also serve as a valuable *experimental* tool to assess the importance of the aforementioned gauche interactions for 2'-alkoxy-DNA/RNA duplex stability and thus further our understanding of the parameters that determine the strength of nucleic acid-nucleic acid interactions.

The above considerations have led us to investigate the hybridization properties and nuclease resistance of oligodeoxyribonucleotides incorporating 2'- or 6'-substituted carbocyclic nucleotides and ultimately assess the suitability of such compounds as building blocks for *antisense* oligonucleotides. In this communication we now wish to report on the synthesis of 2'- and 6'-substituted carbocyclic thymidine analogs **1** - **7** (*Fig. 1*) (or appropriately protected derivatives thereof), their effect on DNA/RNA duplex stability, and on the nuclease resistance of oligodeoxyribonucleotides containing 6'-substituted building blocks **3** - **6**.[12] The pendant groups investigated were chosen such as to modulate the overall physico-chemical properties of the corresponding oligonucleotides in different ways (hydrophobic, charged); oligoethylene glycol derivatives **2** and **7** were included because of indications that oligoethylene glycol conjugates of oligonucleotides might exhibit improved cellular uptake properties.[13]

Scheme 1

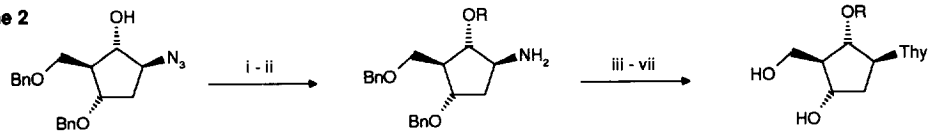


i: TIPSi-Cl_2, DMF/pyridine 2/1, 0°, 50%. ii: $BnOCH_2Cl$, DBU, CH_3CN, 0° → r.t., 89%. iii: 2,3-dihydro-4H-pyran, camphorsulfonic acid (10 mol-%), dioxane, r.t., 83%. iv: CH_3I (solvent), Ag_2O (10 equiv.), refl., 80%. v: NBu_4F (TBAF)/AcOH 1/1, THF, r.t., 95%. vi: H_2, 10% Pd-C, MeOH, 87%. vii: NBu_4F, THF, r.t., 66%. viii: BnBr, NaH, NBu_4I, THF, 0° → r.t., 87%. ix: 90% CF_3COOH, r.t., 81%. x: $CH_3(OCH_2CH_2)_3Cl$, NaH, NBu_4I, DMF, r.t., 28%. xi: H_2, 10% Pd-C, MeOH, 91%.

As shown in *Scheme 1* compounds **1** and **2** were synthesized *via* carbocyclic ribo-thymidine **8**, which had been prepared from (*1R*, *4S*) 2-azabicyclo[2.2.1]hept-5-en-3-one according to literature procedures.[14] Reaction of **8** with dichloro tetraisopropyl disiloxane (TIPSi-Cl_2)[15] and subsequent protection of the base nitrogen N-3 with the benzyloxymethyl (BOM) group[16] gave partially protected intermediate **9** in 44% yield. Alkylation of **9** with CH_3I in the presence of Ag_2O followed by consecutive removal of the TIPSi- and BOM protecting groups then furnished **1** in 66% overall yield (based on **9**). Due to the lability of the TIPSi-protecting group under strongly basic conditions, alkylation of the 2'-OH group with $CH_3(OCH_2CH_2)_3Cl$ in the presence of NaH required prior exchange of the TIPSi-group for two benzyl protecting groups. This was achieved *via* conversion of **9** into its 2'-tetrahydropyranyl (THP) ether **10**, TIPSi-cleavage with TBAF, alkylation with BnBr/NaH and finally removal of the THP group by treatment with 90% CF_3COOH. Alkylation of the 2'-OH group followed by concomitant hydrogenolytic removal of all protecting groups then furnished **2**.

Compounds **4**, **6**, and **7** as well as **3a** and **5a** were prepared from the known homochiral azido alcohol **10**[17] (*Scheme 2*). **3a** and **5a** serve as protected precursors of **3** and **5** for the purpose of

Scheme 2



10

11: R = Bz **12**: R = CH_3
13: R = $(CH_2)_4$N=Pth **14**: R = $(CH_2)_3$Ph
15: R = $(CH_2CH_2O)_2CH_3$

3a: R = Bz **4**: R = CH_3
5a: R = $(CH_2)_4NHCOCF_3$ **6**: R = $(CH_2)_3$Ph
7: R = $(CH_2CH_2O)_2CH_3$

i: **11**: Bz-Cl, Et_3N, Et_2O, 82%; **12**: CH_3I, NaH, THF, 84%; **13** - **15**: R-Br, NaH, THF, 61% - 76%. ii: H_2, Lindlar-catalyst, 81% - 100%. iii: $CH_3OCH{=}C(CH_3)CONCO$, CH_2Cl_2, - 60° → r.t., 92% - 100%. iv: 0.2N HCl EtOH/H_2O 9/1, refl., 82% - 98%. For **5a**: v: H_2N-NH_2 x H_2O, EtOH, refl.. vi: $(CF_3CO)_2O$, Et_3N, DMF, 78% (2 steps). vii: H_2, 10% Pd-C, 84% - 96%.

oligonucleotide synthesis; upon final deprotection of the completed oligonucleotide with ammonia the 6'-substituents of **3a** and **5a** are converted to the free hydroxyl and aminobutyl groups in **3** and **5**, respectively. The syntheses involved acylation or alkylation of **10** with the appropriate electrophiles followed by reduction of the azide functionality by catalytic hydrogenation over Lindlar's catalyst to furnish intermediates **11** - **15**. Elaboration of the primary amino group in **11** - **15** into the heterocyclic base moiety was achieved according to known procedures[18] and the syntheses were then completed by the subsequent removal of the Bn-protecting groups by catalytic hydrogenation over 10% Pd-C (**3a**, **4**, **6**, **7**). In the case of **5a** the phthaloyl (=Pht) protecting group on nitrogen was exchanged for a trifluoro acetyl group prior to the final hydrogenation step.

The data for the *RNA-binding* properties of oligonucleotides containing substituted carbocyclic nucleosides **1** - **7** are summarized in *Table 1*.[19] As indicated by the negative ΔT_m/mod. values for the majority of sequences investigated, the incorporation of *2'-alkoxy* substituted building blocks **1** and **2** into the DNA strand of a DNA/RNA heteroduplex generally causes a decrease in duplex stability (except for sequence **II**).[20] These findings contrast with the effects of 2'-alkoxy substituents on the RNA-binding affinity of oligodeoxyribonucleotides (*vide supra*)[9] and may thus originate in the lack of the gauche effect between the 2'-hetero-substituent and the furanose oxygen in carbocyclic

Table 1: Relative RNA-binding affinities of modified oligodeoxyribonucleotides containing 2'- or 6'-substituted carbocyclic thymidines **1** - **7** (ΔT_m-values/modification (°C)).[a]

Sequence:[b]	**I** (63.9[c])	**II** (52.3[c])	**III** (61.8[c])	**IV** (61.7[c])	**V** (48.0[c])
Modified dT					
carbo-dT[d]	+ 0.2 (+ 0.3[e])	+ 0.4[f]	-	-	+ 0.25[g]
1	- 1.9	+ 1.1	-	- 0.7	- 1.0
2	- 2.9	+ 0.2	- 1.1	- 2.6	non coop.[h]
3	-	- 0.8 (+ 0.4[i])	- 0.4	+ 0.2	+ 0.8
4	+ 0.2	- 2.0	- 1.3	- 0.7	- 0.9
5	- 1.8	- 1.1	- 1.4	-	- 1.9
6	- 2.8	- 4.0	- 2.8	- 1.7	non coop.[h]
7	-	- 1.8 (- 1.9[i])	-	-	-

[a]Difference in melting temperature (T_m) between the modified DNA/RNA duplex and the unmodified wild-type (WT) duplex per modification ($\Delta T_m = T_m - T_m(WT)$). T_m's were determined in 10 mM phosphate buffer, pH 7, 100 mM Na^+, 0.1 mM EDTA. T_m-values are ± 0.5°. For details see ref. 9b; [b]Sequences are: **I**: 5'-CTCGTACC**t**TTCCGGTCC-3'; **II**: 5'-TTTT**t**CTCTCTCTCT-3'; **III**: 5'-**t**CCAGG**t**G**t**CCGCA**t**C-3'; **IV**: 5'-CTCGTAC**ttttt**CCGGTCC-3'; **V**: 5'-GCG**tttttttttt**GCG-3'; **t** = modified thymidine; [c]T_m of the corresponding wild-type duplex in °C; [d]unsubstituted carbocyclic thymidine; [e]For 5'-**ctc**G**tacctttcc**GG**tc**C-3' (ref. 3c; **c**, **a** = carbo dC, dA); [f]For 5'-**ttttctctctctc**T-3' (this work); [g]For 5'-(**t**)$_8$-3' (this work); [h]no cooperative melting observed (T_m < 20°); [i]For 5'-**ttttc*tc*tc*tc*tc***T-3' (c* = 5-methyl dC analog).

nucleotides.[11] In the absence of such directing electronic interactions steric factors will favor an *equatorial* orientation of the 2'-substituent (*2'-endo* conformation) and thus lead to a low degree of conformational preorganization in terms of A-type DNA/RNA duplex formation (which requires a 3'-endo conformation with the 2'-substituent in an *axial* orientation).[6] Similar arguments have been invoked to rationalize the preference of 2'-α-alkyl substituted 2'-deoxyribonucleosides for a 2'-endo conformation (no gauche effect between the furanose oxygen and a 2'-*alkyl* substituent) as well as their unfavorable effects on DNA/RNA duplex stability.[8] Consistent with the results obtained in the present study for **1** and **2**, DNA/RNA duplex destabilization by 2'-α-alkyl substituted 2'-deoxyribonucleotides has also been found to increase with increasing size of the 2'-substituent.

Reduced RNA-binding affinity is also observed for oligonucleotides incorporating *6'-alkoxy* substituted carbocyclic building blocks **4** - **7** (*Table 1*). Again DNA/RNA duplex destabilization is least pronounced for a methoxy substituent (**4**), while larger and/or more hydrophobic groups lead to a further decrease in duplex stability. This is particularly evident from the hybridization properties of sequence **V**, where T_m-values of 39.5° and 29° (ΔT_m = 10.5°) are observed for the two versions containing either **4** or **5**, respectively; incorporation of **6** even causes a complete loss of RNA-binding affinity for this sequence. In view of the very limited data base for compound **7** its effect on DNA/RNA duplex stability is rather difficult to classify with respect to **4**, **5**, and **6**. However, a ΔT_m/mod.-value of - 1.8° for an almost completely modified version of sequence **II** (*Table 1*) points to a very similar degree of duplex destabilization for **7** and **5** (with a ΔT_m/mod.-value of - 1.9° being observed for **5** in sequence **V**).

As indicated above, DNA/RNA duplex destabilization by 6'-alkoxy substituents is unlikely to be caused by inevitable steric interferences of the 6'-substituent with parts of the actual duplex structure. In addition, 6'-alkoxy groups are not expected to severely restrict the conformation of the cyclopentane moiety such as to effectively oppose A-type duplex formation. In fact, preliminary studies on the solution conformation of **4** in D_2O indicate the molecule to preferentially adopt a 4'-exo conformation (with the 6'-methoxy group in a pseudo-equatorial orientation), which is closely related to the 3'-endo conformation of the sugar moieties of A-type double helices. The most plausible explanation that can presently be invoked for the adverse effects of **4** - **6** on DNA/RNA duplex stability may thus be the unfavorable solvation of their solvent exposed, hydrophobic 6'-substituents.[21] However, additional factors are likely to be involved, especially for the diethylene glycol substituent in **7**,[22] and the presence of alkoxy groups at the 6'-α position of carbocyclic nucleotides may in fact be intrinsically unfavorable for DNA/RNA duplex formation, independent of the exact nature of the substituent (*vide infra*).

The effects of *6'-hydroxy* carbocyclic thymidine **3** on DNA/RNA duplex stability are distinctly different from those of alkoxy substituted building blocks **4** - **7**. Although incorporation of **3** into the DNA strand of a DNA/RNA heteroduplex at isolated sequence positions (sequences **II** and **III**, *Table 1*) also results in a modest destabilization of the hybrid structure, the effect is clearly less pronounced than even that of **4** (T_m-values of 60.6° and 57.1° are observed for sequence **III** containing **3** or **4**, respectively). Moreover, the presence of stretches of contiguous building blocks **3** as part of an oligodeoxyribonucleotide leads to an *increase* in the thermodynamic stability of the corresponding DNA/RNA hybrids (as compared to the respective wild-type duplexes). This is particularly obvious for sequence **V** and an almost completely modified version of sequence **II**, where T_m-values rise by 8° and 6.5°, respectively, upon incorporation of **3**.

While the more favorable hybridization behavior of oligonucleotides containing building blocks **3** rather than **4** - **7** at isolated sequence positions may simply arise from more favorable interactions with the surrounding solvent (i. e. improved solvation),[23] the further increase in duplex stability due to the presence of stretches of contiguous modifications may be indicative of a structure stabilizing hydrogen bonding network involving OH-groups of adjacent carbocyclic nucleotide units.

It is important to note that the data in *Table 1* do not allow any firm conclusions to be drawn regarding the relative RNA-binding affinity of fully modified oligonucleotides that would be entirely composed of either 6'-unsubstituted or 6'-hydroxy carbocyclic 2'-deoxyribonucleotides, respectively.

Table 2: Effect of 6'-substituted carbocyclic nucleotides on the nuclease resistance of 5'-d(TCC AGG TGT CCG **ttt** C)-3' in 10% heat-inactivated fetal calf serum (FCS).[a]

t	R_1[b]	R_2[b]	$T_{1/2}$ (h)[c]	$T_{1/2}$ (WT) (h)[d]	$T_{1/2}/T_{1/2}$ (WT)
carbo-dT	H	H			2.5[e]
3	H	OH	14	2	7
4	H	OCH_3	20	2	10
5	H	$O(CH_2)_4NH_2$	> 48	2	> 24
6	H	$O(CH_2)_3Ph$	> 48	2	> 24

[a]For experimental details cf. ref. 24. [b]Cf. Fig. 1. [c]Time period after which the sum of the concentrations of the full length oligonucleotide and the truncated species generated by enzymatic removal of the natural dC residue at the 3'-end corresponds to 50% of the initial oligonucleotide concentration. [d]$T_{1/2}$ for the corresponding wild-type oligonucleotide (**t** = natural dT) obtained with the same batch of FCS. [e]Ref. 3c.

It appears, however, as if both types of structural modifications lead to a comparable degree of DNA/RNA duplex stabilization (this is also supported by other data not shown here), thus rendering the attachment of a hydroxyl group to the 6'-position of carbocyclic nucleotides a virtually neutral structural change with respect to RNA-binding affinity (in the context of a fully modified sequence). Taken together, the hybridization properties of **3** containing oligonucleotides would then imply that even the attachment of a free hydroxyl group (and, by analogy, also a 6'-alkoxy group) to the 6'-α-position of carbocyclic nucleotides *intrinsically* disfavors DNA/RNA duplex formation, although this effect can be compensated for by other favorable interactions for adjacent modified building blocks.

The effect of 6'-substituted carbocyclic nucleotides on the stability of oligodeoxyribonucleotides against degradation by 3'-exonucleases has been investigated for the sequence 5'-d(TCC AGG TGT CCG **ttt** C)-3' in 10% heat-inactivated fetal calf serum (FCS) (where **t** designates a modified building block).[24] As shown in *Table 2* the attachment of an additional substituent to the 6'-α-position of carbocyclic thymidine in all cases investigated (**3** - **6**) leads to increased nuclease resistance. This confirms our original working hypothesis, which assumed that the introduction of substituents in positions that are unsubstituted in the natural enzyme substrate (natural DNA) would lead to less efficient processing by nucleolytic enzymes, i. e. lower susceptibility of the modified oligonucleotides to enzymatic cleavage of the phosphodiester backbone. Not too surprisingly, enzymatic stability increases with increasing steric bulk of the 6'-substituent, with **5** and **6** providing a dramatic improvement in nuclease resistance over the corresponding wild-type oligonucleotide.

In conclusion, there exists an inverse relationship between the effects of **3** - **6** on nuclease stability and RNA-binding affinity of the corresponding oligonucleotides, i. e. the highest degree of nuclease resistance is conferred by substituents causing the most significant decrease in DNA/RNA duplex stability. This finding imposes severe limits on the general applicability of compounds **3** - **7** as building blocks for antisense oligonucleotides, as the anticipated superior nuclease resistance of fully modified sequences composed of building blocks **5** - **7** (and probably also **1**) will be more than offset by their inferior RNA-binding affinity. On the other hand, the metabolic stability of oligonucleotides based on **3** or **4** is unlikely to suffice for biological applications at reasonably low concentrations, this problem being additionally compounded in the case of **4** by its unfavorable effect on RNA-binding affinity. However, in view of its beneficial effect on DNA/RNA duplex stability, the incorporation of **3** into nuclease resistant backbone modified oligonucleotides (e.g. phosphorothioates) may still constitute a promising approach to highly potent antisense molecules.

Acknowledgements: We thank Drs. U. Pieles and D. Hüsken for oligonucleotide synthesis and purification and DH and Dr. S. M. Freier for T_m-measurements.

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19. For the purpose of oligonucleotide synthesis **1** - **7** were converted to the corresponding 5'-O-(4,4'-dimethoxytrityl) (DMTr) 3'-(2-cyanoethyl-N,N-diisopropylamino) phosphite (Sinha, N. D.; Biernat, J.; McManus, J.; Köster, H. *Nucleic Acids Res.* **1984**, *12*, 4539). Oligonucleotides were synthesized on an ABI 390 DNA synthesizer using standard phosphoramidite chemistry, except that coupling times of 10 - 12 min were routinely employed for modified building blocks (cf. "Oligonucleotide synthesis - a practical approach", Gait, M. J., Ed., IRL Press, Oxford, 1984). The 5'-DMTr-protected products were purified by RP-HPLC and according to gel electrophoresis or capillary electrophoresis (CE) the fully deprotected oligonucleotides were at least 95% pure. For sequence **II** the intact incorporation of the modified building blocks was also verified by MALDI-TOF MS (Pieles, U.; Zürcher, W.; Schär, M.; Moser, H. E. *Nucleic Acids Res.* **1993**, *21*, 3191).
20. Given the error limits of the T_m-measurements the ΔTm value for **1** in sequence **II** should reflect a true increase in duplex stability, which may be a sequence specific phenomenon. In contrast, the increase in T_m for **2** in sequence **II** does not necessarily signify a stabilizing effect; however, the fact remains that duplex destabilization caused by **2** would clearly be least pronounced for this sequence.
21. Despite the presence of a positively charged head group the aminobutyl side chain in **5** is also rather lipophilic in nature (4 hydrophobic methylene units). We have not addressed the question whether the different behavior of **5** and **6** may have to do with favorable charge-charge interactions between the positively charged 6'-substituent in **5** and the negatively charged phosphate backbone.
22. Polyethylene glycols (PEG's) are known to strongly improve the aqueous solubility of covalently attached hydrophobic peptides, thus indicating the PEG chain to be very well solvated in aqueous solution: See, e. g.: Pillai, V. N. R.; Mutter, M. *Acc. Chem. Res.* **1981**, *14*, 122.
23. Analysis by ^{1}H-NMR indicates the solution conformation of **3** and **4** to be very similar, changes in the conformation of the cyclopentane moiety thus being unlikely to be responsible for the different RNA binding affinities of oligonucleotides containing **3** or **4** at isolated sequence positions.
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(*Received in Belgium* 28 *September* 1994; *accepted* 23 *December* 1994)