Inhibition of Thyroid Hormone Uptake by Calcium Antagonists of the Dihydropyridine Class

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A series of substituted 4-phenyl-1,4-dihydropyridines **2a**−**m** was tested for their inhibitory effects on L-triiodothyronine (L-T_3) uptake by human HepG2 hepatoma cells. The most potent compounds were the nitro-substituted derivatives 2,6-dimethyl-4-(4′-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid 3-ethyl ester 5-methyl ester (**2m**) and the well-known calcium antagonists nitrendipine (**2k**) and nifedipine (**2j**) with an uptake inhibition between 80.5 and 85.8% at an application dose of 10^{-5} M. On the basis of a theoretical conformational analysis (*ab initio* MO theory, molecular mechanics, molecular dynamics) of the dihydropyridine derivatives, a unifying stereochemical concept was derived postulating an angular arrangement of the two rings where the phenyl ring of the calcium antagonists, which corresponds to the outer phenyl ring of the thyroid hormones, is bisecting the dihydropyridine ring as a prerequisite for inhibitory potency. This model includes also inhibitors of the *N*-phenylanthranilic acid type. The interaction of the calcium antagonists with transthyretin (TTR) is discussed in relation to thyroid hormones. The influence of hydrophobicity was estimated by the experimental determination of the 1-octanol/water partition coefficients.

Introduction

The entry of the thyroid hormone L-triiodothyronine (L-T_3, **1**) (Figure 1) from blood into the cell is an essential step in the complex process of thyroid hormone action.[1,2] The detailed molecular mechanism of this entry is not yet clear, although numerous experimental studies show stereospecific, saturable, and energy- and temperature-dependent uptake, suggesting that the passage of thyroid hormones into somatic cells is the result of a receptor-mediated process.[3−8] Although cell membrane binding sites for L-T_3 have been identified,[9−13] they were not convincingly characterized. Thus, their role in uptake remains still uncertain. Several functionally unrelated classes of compounds, *e.g.* dihydropyridine calcium channel blockers **2** (Figure 1), nonsteroidal antiinflammatory drugs of the *N*-phenylanthranilic acid type **3** (Figure 1), and calmodulin antagonists from the naphthalene sulfonamide class, are able to inhibit the uptake of L-T_3 in rat H4 and human HepG2 hepatoma cells.[14,15]

Furthermore, benzodiazepines are described as inhibitors of the postulated thyroid hormone transporter.[16,17] Recently, Yokoyama *et al.*[18] reported that a series of oxamic acid and acetic acid derivatives of thyronine showed *in vitro* binding to rat liver nuclear and rat membrane L-T_3 receptors. The inhibitory action of the above-mentioned compounds on the L-T_3 uptake can by no means be related to their well-known pharmacological activities. Thus, former studies provided no hints for any calcium-dependent regulation of the L-T_3 uptake consistent with the absence of voltage-dependent calcium channels in these cells.[19,20] Compounds acting as L-T_3 antagonists could be useful pharmacological tools to investigate the thyroid hormone action as well as potent therapeutic agents for the effective treatment of disorders caused by thyroid hormone excess. Therefore, we tested a series of substituted 4-phenyl-1,4-dihydropyridines for their potency of L-T_3 uptake inhibition in human HepG2 cells.

The interaction between biologically active compounds and their receptors is based on special structural and physicochemical properties. There are numerous reviews on structure−function relationships in the class of thyroid hormones.[21−29] The structure elements necessary for effective binding and activity are well defined. On the basis of X-ray data for complexes of the hormones and transthyretin (TTR), correlations between the molecular structure and binding affinities were discussed.[21,30,31] Considering these results, we thought to explain our measured inhibitory actions of the 4-phenyl-1,4-dihydropyridines within a unifying concept by comparison of their structural and physicochemical properties with those for the thyroid hormones. In view of such a concept, we consider additionally the class of *N*-phenylanthranilic acid derivatives, which were found to be L-T_3 uptake inhibitors by us in a former study.[15]

Results and Discussion

1. Inhibition Studies. We investigated the inhibitory potency of the 4-phenyl-1,4-dihydropyridines **2a**−**m** (for structures, see Table 1) in human HepG2 hepatoma cells. These compounds vary in the substitution both of the phenyl ring and the type of ester groups at the 3- and 5-positions of the dihydropyridine ring. Nifedipine **2j** and nitrendipine **2k** were already tested in a previous study in rat H4 hepatoma cells[32] and proved to be the most potent uptake inhibitors, thus allowing a compari-

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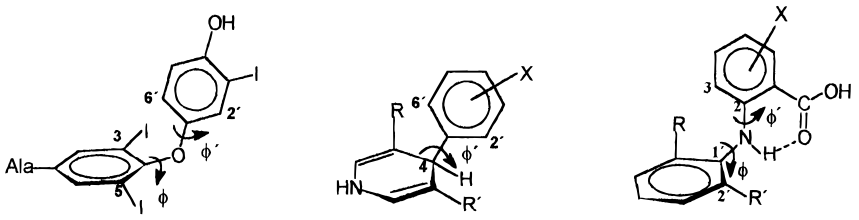
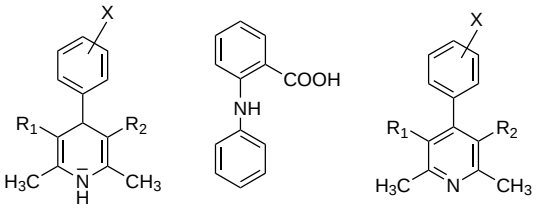


1 **2** **3**

Figure 1. Basic systems of thyroid hormones (L-T_3) **1**, dihydropyridine calcium antagonists **2**, and *N*-phenylanthranilic acids **3** in their minimum conformations.

Table 1. Inhibition Data and Partition Coefficients for the Dihydropyridines **2a**−**m**, *N*-Phenylanthranilic Acid **3**, the Pyridine Derivative **4**, Diphenyl Ether, and Diphenylamine



2a-m **3** **4**

compd	R^1	R^2	X	% I[a]	log*P*[b]	c log*P*[c]
2a	$COOC_2H_5$	$COOC_2H_5$	3′-I	33.2	4.48	4.38
2b	$COOC_2H_5$	$COOC_2H_5$	4′-I	26.8	4.70	4.38
2c	$COOC_2H_5$	$COOC_2H_5$	3′-OH	21.6	4,03	2.68
2d	$COOC_2H_5$	$COOC_2H_5$	3′-CH_3	29.7	3.76	3.69
2e	$COOCH_3$	$COOC_2H_5$	2′-CF_3	23.1	3.82	3.82
2f	$COOCH_3$	$COOC_2H_5$	3′-CF_3	19.6	3.90	3.82
2g	$COOC_2H_5$	$COOC_2H_5$	2′-OCH_3	22.3	3.19	3.19
2h	$COOC_2H_5$	$COOC_2H_5$	3′-OCH_3	17.5	3.22	3.19
2i	$COOCH_3$	$COOC_2H_5$	2′-NO_2	41.4	2.51	3.65
2j	$COOCH_3$	$COOCH_3$	2′-NO_2	80.5	2.06	2.21
2k	$COOCH_3$	$COOC_2H_5$	3′-NO_2	81.8	−	3.16
2l	CN	CN	3′-NO_2	53.8	1.45	1.75
2m	$COOCH_3$	$COOC_2H_5$	4′-NO_2	85.8	3.60	2.61
3				20.5	3.08	2.96
4	$COOC_2H_5$	$COOC_2H_5$	3′-NO_2	28.4	3.10	3.45
diphenyl ether				22.0	3.83	3.85
diphenyl amine				20.9	2.94	3.38

[a] Application dose 10^{-5} M. [b] Determined by RP-HPLC. [c] Calculations are based on the PALLAS program.

son between both cell models to be made. In order to verify structure−activity relationships, the simple diphenyl ether molecule was also included. In the corresponding way, diphenylamine was tested in relation to the *N*-phenylanthranilic acid series of our former study[15] to find support for the explanation of the activity and the development of a common stereochemical concept for both classes of compounds. To further assess some ideas of structure-activity relationships in the dihydropyridine series, we also tested the 4-phenylpyridine derivative **4** (Table 1).

The results of the inhibition studies are presented in Table 1. The investigated dihydropyridines showed L-T_3 uptake inhibition in the range 17.5−85.8% at a dose of 10^{-5} M. The most potent inhibitors in our test system were the 4′-nitro derivative **2m**, nifedipine (**2j**), and nitrendipine (**2k**) with IC_{50} values of 4.8×10^{-6}, 2.2×10^{-6}, and 1.2×10^{-6} M, respectively. Our IC_{50} values for nifedipine and nitrendipine in human HepG2 cells are comparable with the former results in rat H4 cells.[32] This indicates that both cell models are approximately equivalent. Diphenyl ether and diphenylamine as well as the pyridine derivative **4** proved to be poor inhibitors of L-T_3 uptake. The comparison between the inhibition data for the calcium antagonists and those for a series of *N*-phenylanthranilic acids[15] shows that the dihydropyridines are generally more potent inhibitors.

2. Structure−Activity Relationships. 2.1. Conformational Aspects. The conformational requirements for the thyroid hormones to achieve good binding properties are well understood and documented.[21−29] With respect to the dihydropyridine calcium antagonists and the *N*-phenylanthranilic acid derivatives of our former study,[15] a comparison between their conformation and that of the diphenyl ether moiety in the thyroid hormones could especially be instructive. Numerous structure determinations of thyroid hormone derivatives (for a review, *cf.* ref 21) indicate an approximate skewed form **1a** (Figure 2) with torsion angles ϕ of about ±90° and ϕ' of about 0° in the diphenyl ether part. Here, the rotation angles ϕ and ϕ' are defined *via* the atoms C5−C4−O−C1′ and C4−O−C1′−C6′, respectively (Figure 1). This contrasts with the simple diphenyl ether molecule which prefers a propeller-like conformation **1b** (Figure 2) with experimentally determined torsion angles of $\phi = \phi' = 37°$.[21] The space-filling *ortho* iodine substituents in the thyroid hormones enforce quite obviously a perpendicular orientation of the phenyl rings to each other, where the outer ring bearing the 4′-hydroxy group is bisecting the plane of the inner phenyl ring (Figure 1).

Structure data obtained on the basis of *ab initio* MO theory at the HF/6-31G* level may illustrate these aspects (Table 2). Thus, torsion angles of $\phi = \phi' = 47.6°$ were estimated for diphenyl ether corresponding to the propeller-like conformation **1b** (Figure 2). The calculated rotational barriers of 0.1 and 10.9 kJ·mol^{-1} related to the skewed form **1a** and the perpendicular form **1c** with $\phi = \phi' = 90°$ (Figure 2) are small. The different conformation and the high flexibility could be responsible for the low inhibitory potency found for diphenyl ether in our experiments. Introduction of chloro substituents (a 6-31G* basis set is not defined for iodine) at the *ortho* positions of one phenyl ring of diphenyl ether already leads to the preference of the skewed conformation **1a** postulated as active conformation (Table 2). The rotational barrier related to **1c** is 23.4 kJ·mol^{-1}. In fact, such derivatives show higher binding affinity even if it is still lower than that of the iodinated compounds.[21,22]

The examination of the conformation of model compounds of the dihydropyridine calcium antagonists at the same HF/6-31G* level of *ab initio* MO theory shows remarkable similarities to the preferred thyroid hormone conformations (for HF/STO-3G calculations on dihydropyridine calcium antagonists, *cf.* refs 33, 34). The

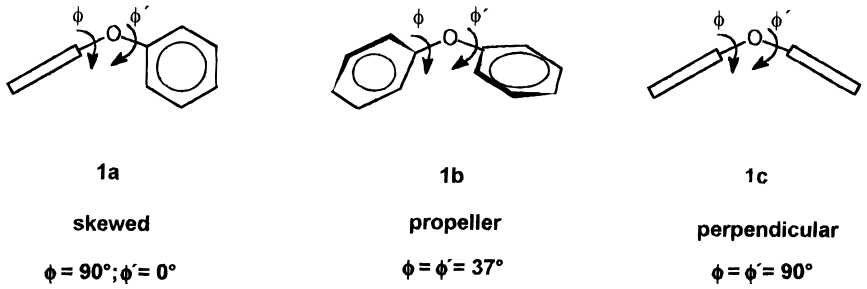


Figure 2. Selected conformations of the diphenyl ether system (for torsion angle conventions, *cf.* Text and Figure 1).

Table 2. *Ab Initio* HF/6-31G* Torsion Angles for the Minimum Conformations of Selected Model Compounds of L-T_3, Dihydropyridine, and *N*-Phenylanthranilic Acid Type

compd	$\phi^{a,b}$	$\phi'^{a,b}$
diphenyl ether[c]	47.6	47.6
2,6-dichlorodiphenyl ether[d]	92.6	1.9
4-phenyl-1,4-dihydropyridine[e]		0.0
4-phenyl-1,4-dihydropyridine-3,5-dicarboxylic acid[f]		0.0
diphenylamine[g]	29.9	29.9
2,6-dichlorodiphenylamine[h]	78.5	−0.9
N-phenylanthranilic acid[i]	58.4	−9.2

[a] Angles in degrees. [b] Torsion angles in the diphenyl ether derivatives, ϕ C5−C4−O−C1′, ϕ' C4−O−C1′−C6′; in the *N*-phenylanthranilic acids, ϕ: C2′−C1′−N−C2, ϕ' C1′−N−C2−C3; in the dihydropyridines ϕ' H4−C4−C1′−C2′, see Figure 1. [c] E_T = −535.098 54 au. [d] E_T = −1452.888 53 au. [e] E_T = −477.370 00 au. [f] E_T = −852.621 22 au. [g] E_T = −515.271 74 au. [h] E_T = −1433.063 79 au. [i] E_T = −702.897 61 au.

perpendicular orientation of the phenyl and the dihydropyridine rings to each other corresponding to the skewed form of the thyroid hormones represents the minimum conformation even for the unsubstituted 4-phenyl-1,4-dihydropyridine (R = R′ = X = H in **2**, Figure 1). The dihydropyridine ring exhibits a boatlike conformation.[33,35] To get formal correspondence of the rotation angle values of the calcium antagonists and the thyroid hormones, the phenyl ring torsion angle in the dihydropyridines is related to ϕ' in **1** and defined *via* the atoms H4−C4−C1′−C2′ in **2** (Figure 1). Thus, a value of ϕ' = 0° or, alternatively, 180° in the phenyldihydropyridines corresponds to a conformation where the phenyl ring is bisecting the dihydropyridine ring. The rotation barrier around the central CC single bond amounts to 18.2 kJ·mol^{-1}. Introduction of two carboxy groups at the 3- and 5-positions of the dihydropyridine ring as model substituents for the ester groups occurring in the calcium antagonists leads to the same minimum conformation with ϕ' = 0° (Table 2). The phenyl ring rotation barrier is slightly increased by 3.4 kJ·mol^{-1}. The theoretical results are in complete agreement with the results from numerous X-ray structure determinations on dihydropyridine calcium antagonists clearly indicating the skewed conformation.[36−40]

In order to search for a unifying structure concept for L-T_3 uptake inhibitors the conformations of the diphenylamine molecule and some of its derivatives were also examined. The *ab initio* data show a conformation with torsion angles of $\phi = \phi'$ = 29.9° for diphenylamine which may well be compared with the propeller-like diphenyl ether conformation (Table 2). The measured low inhibitory potency of diphenylamine (Table 1) may be due to this arrangement differing from the thyroid hormone minimum conformation. As in the diphenyl ether system, a more perpendicular ring arrangement is supported by larger *ortho* substituents. Thus, the calculated torsion angles in 2,6-dichlorodiphenylamine are ϕ = 78.5° and ϕ' = -0.9° (Table 2). Although the conformation of *N*-phenylanthranilic acid **3** with torsion angles of ϕ = 58.4° and ϕ' = −9.2° is closer to the structure of thyroid hormones, the inhibitory potency still remains low (Tables 1 and 2). Interestingly, the minimum conformation of the *N*-phenylanthranilic acid shows a stabilizing hydrogen bond between the carboxy group and the NH group linking the two phenyl rings as indicated in Figure 1. The existence of this hydrogen bond may be important for the fixation of a more skewed orientation of the rings induced by *ortho* substituents. Thus, 2′,6′-dichloro-*N*-phenylanthranilic acid represents a relatively potent L-T_3 uptake inhibitor in this series as shown in our former study which also indicates a considerable loss of inhibitory potency if the carboxy group is missing even for the same substituent pattern of potent *N*-phenylanthranilic acids.[15]

Structure modifications of the ether linkage of the thyroid hormones show the activity and affinity retained when replacing the oxygen atom by NH or even a CH_2 group.[21,22,24] Thus, the inhibitory potency of the *N*-phenylanthranilic acid series is not surprising provided that the skewed conformation may at least approximately be realized and kept fixed. The structure of the 4-phenyldihydropyridines illustrates that the ether linkage may even be missing without a loss of affinity. Apart from the structural prerequisites mentioned before, the angular orientation of the two rings seems to be an essential point for activity. The considerable decrease of inhibitory potency found for the 4-phenylpyridine derivative **4** (Table 1), where the rings are in a linear arrangement, although considerably twisted by 65.3°, indicates the importance of this structural aspect for activity additionally supported by the missing activity of biphenyl analogues of thyroid hormone derivatives.[22,24]

In Table 3, structure information resulting from molecular mechanics calculations is given for all dihydropyridine calcium antgonists **2a**−**m** which were tested in our uptake inhibition study. It becomes visible that the skewed conformation is realized in all derivatives as already predicted by the *ab initio* data. Since the 2′/6′- and 3′/5′-positions, respectively, become nonequivalent with respect to the dihydropyridine ring, proximal (near) and distal (away) conformations of the phenyl ring can be differentiated for substituted derivatives as found in the thyroid hormones, too.[21] The calculations show a slight preference of the distal conformation in most cases (Table 3). As expected, the rotational barrier separating the distal and proximal conformers is higher for the *ortho*-substituted derivatives in comparison to *meta*- and *para*-substituted compounds.

Table 3. Torsion Angles of the Minimum Conformations, Energy Differences between the Distal and Proximal Conformers, Rotation Barriers, and Molecular Volumes for the Dihydropyridines **2a**−**m** and the Pyridine Derivative **4** on the Basis of Molecular Mechanics Employing the CHARMm Force Field

compd[a]	ϕ'[b,c]	ΔE[d,e]	ΔE_{rot}[d,f]	V[g]
2a	−2.2/−4.3	−4.6	25.9	355.2
2b	−3.9		23.4	353.0
2c	−3.4/−4.6	−2.1	25.1	331.1
2d	−2.9/−4.0	−2.1	24.3	336.3
2e	−0.5/−9.6	−3.3	62.3	332.0
2f	−6.3/−6.2	−3.8	25.1	329.9
2g	−2.3/−2.7	−0.4	39.3	341.9
2h	−3.3/−4.7	−2.1	24.7	347.7
2i	−3.1/−11.5	−13.4	57.7	326.5
2j	−7.5/−11.8	−13.4	55.2	310.2
2k	−8.4/−6.8	−2.9	25.1	329.6
2l	0./0.	0.8	23.8	259.3
2m	−6.1		23.0	329.3
4	65.3			316.5

[a] For the structures, see Table 1. The ester carbonyl groups are in *syn,syn* orientation to the double bonds of the dihydropyridine ring. [b] Angles in degrees. [c] First value for the distal, second value for the proximal conformer. [d] Energies in kJ·mol^{-1}. [e] Energy difference distal − proximal. [f] Rotation barrier related to the distal conformer. [g] Molecular volumes in Å^3.

The strong preference of the skewed conformation can well be confirmed by the results from molecular dynamics simulations on the two very potent L-T_3 uptake inhibitors nifedipine (**2j**) and 2′,6′-dichloro-*N*-phenylanthranilic acid in comparison to 3,5,3′-triiododiphenyl ether as a model compound for L-T_3. Thus, the torsion angles from 100 ps trajectories are $\phi = 91.3 \pm 13.3°$, $\phi' = 0.9 \pm 19°$ for the triiododiphenyl ether, $\phi' = -0.1 \pm 15.2°$ for nifedipine, and $\phi = 89.1 \pm 22.1°$, $\phi' = -0.2 \pm 29.1°$ for the *N*-phenylanthranilic acid derivative.

2.2. Substituent Influence and Parameters of Receptor−Ligand Interaction. When considering the influence of substituents on the inhibitory strength in the dihydropyridine series, the increase of inhibition by nitro groups on the phenyl ring is most striking. Thus, compounds such as nifedipine (**2j**) and nitrendipine (**2k**) (*cf.* also ref 41) represent very good inhibitors of the L-T_3 uptake. The most active compound in our test model was the 4′-nitro derivative **2m** with an uptake inhibition of 86% at a concentration of 10^{-5} M. The other dihydropyridine compounds are rather poor inhibitors, although still more potent than the corresponding *N*-phenylanthranilic acid derivatives bearing the same substituents.[15] Interestingly, the inhibitory potency of the iodine-substituted derivatives is considerably lower than that of the nitro-substituted compounds despite their closer similarity to L-T_3.

The length of the alkyl side chains of the ester groups has some influence on the strength of inhibition. Thus, the comparison between the dimethyl ester derivative nifedipine **(2j)** and compound **2i**, where one of the dimethyl ester groups is replaced by an ethyl ester group, shows about 50% loss of the inhibitory activity. Obviously, shorter ester groups seem to be favored, while replacement of the ester groups by nitrile groups as in **2l** decreases inhibitory potency.

The *ab initio* and molecular mechanics structure data for our dihydropyridine and *N*-phenylanthranilic acid series provide some ideas on structural prerequisites for inhibitory potency when compared with the structure of thyroid hormones. However, the considerable variation of the inhibition data in Table 1 despite the realization of the skewed conformation in all calcium antagonists turns the attention to further factors possibly influencing the binding affinity.

For this purpose, it seemed to be important to compare the size of dihydropyridines and *N*-phenylanthranilic acids in their minimum conformations with the diphenyl ether part of thyroid hormones. The calculated molecular volumes for the dihydropyridine series are given in Table 3. The data for the *ortho*- and *meta*-substituted derivatives are for the distal substituent orientations. However, similar values were found for the proximal conformation. The comparison with the volume of 308.8 Å^3 for the L-thyroxine (L-T_4) diphenyl ether part shows rather good agreement for the most potent nitro-substituted inhibitors **2j**, **2k**, and **2m**, whereas the volume of the 2′,6′-dichloro-*N*-phenylanthranilic acid is with 223.5 Å^3 remarkably smaller. This volume difference could be a reason for the generally lower inhibition potency of *N*-phenylanthranilic acid derivatives. Nevertheless, dihydropyridines with about the same size still differ considerably in their inhibition potency (Tables 1 and 3). Thus, it may be useful to look for differences in the interaction properties of the various derivatives. Information on the electrostatic interaction possibilities can be gained from the electrostatic potentials on the van der Waals surface area of the molecules. In Figure 3, the electrostatic potentials are visualized for the L-T_4 diphenyl ether part, the dihydropyridine derivative **2k**, and the 2′,6′-dichloro-*N*-phenylanthranilic acid. The electrostatic potentials of the three compounds show some differences caused by the different substituent pattern, in particular concerning the outer ring. This may have some consequences for the electrostatic interaction behavior, which may, however, only be characterized when studying the entire protein−ligand complexes (*vide infra*).

In order to estimate the influence of hydrophobicity on the inhibitory potency of the various derivatives, the 1-octanol/water partition coefficients of all members of the dihydropyridine series were experimentally determined by RP-HPLC. Since log *P* values larger than 3.5 could be expected, an indirect method was selected applying a *n*-octadecane/methanol−water partition system.[42] For comparison the 1-octanol/water partition coefficients were calculated using the PALLAS program.[43] The obtained experimental and theoretical values given in Table 1 are in rather good agreement. However, we could not establish a quantitative relationship between hydrophobicity and inhibitory strength in the dihydropyridine series. This fact has to be mentioned since a parabolic relationship between *c*log*P* values and the inhibitory potency was found in the *N*-phenylanthranilic acid series.[15] Rather interesting are the significant differences between the partition coefficients of the nitro- and iodine-substituted dihydropyridines, indicating distinctly lower values for the more potent nitro derivatives than for the iodine compounds.

A better understanding of the substituent influence on the interaction properties of the inhibitors demands consideration of the binding partner. It may be justified to select TTR as a model for the L-T_3 receptor to describe the interaction behavior. On the basis of the X-ray structure of the complex between TTR and L-thyrox-

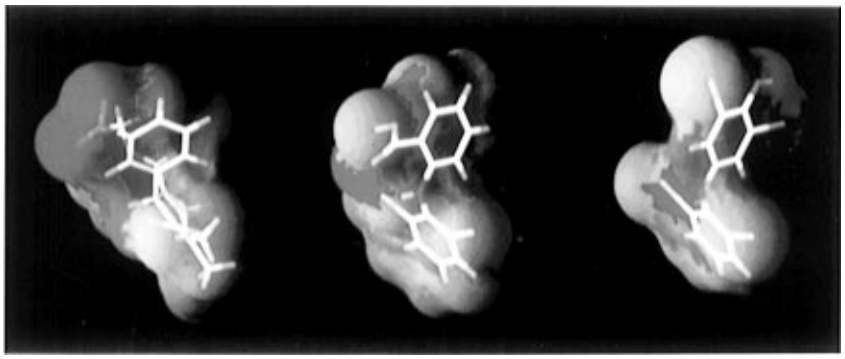


Figure 3. Electrostatic potentials on the van der Waals surface area of the dihydropyridine calcium antagonist **2k** (on the left), 2′,6′-dichloro-*N*-phenylanthranilic acid (middle), and 3,5,3′-triiododiphenyl ether (on the right).

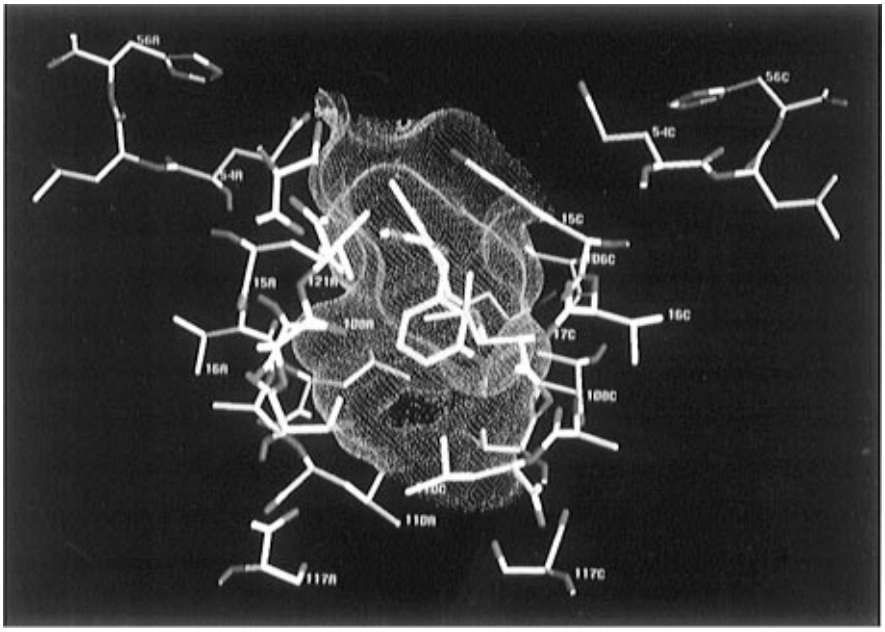


Figure 4. Section of the optimized complex between TTR and 2,6-dimethyl-4-(3′-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid methyl ester (the dotted surface area corresponds to the interior of the protein channel).

ine,[44] several substituted 4-phenyl-1,4-dihydropyridine-3,5-dicarboxylic acid methyl esters and 2′,6′-dichloro-*N*-phenylanthranilic acid were fitted onto the diphenyl ether part of L-T_4 (*cf.* Experimental Section) followed by complete geometry optimization of the protein−ligand complexes. Besides, the transthyretin complex with only the phenyl ether moiety of L-T_4 was considered for comparison. There is a detailed description of the structural basis of the interactions between L-T_4 and TTR which can be referred to when discussing the interaction of the dihydropyridines and other inhibitors.[30] Figure 4 provides a section of the optimized TTR complex with the 3′-nitrodihydropyridine derivative in about the same representation as given for the TTR complex with L-T_4 in ref 30. The dihydropyridines are capable to realize an orientation rather equivalent to L-T_4 with the ester groups in the hydrophobic pockets where the iodine substituents of the inner ring of L-T_4 are normally positioned. The approximately skewed form is retained. The more polar character of the ester groups may be disadvantageous for binding in the very apolar pockets. The limited size of these pockets makes it understandable that methoxycarbonyl groups fit better than ester groups of larger size. The position of the 3′-nitro group corresponds about to the localization of the distal iodine substituent of the outer L-T_4 phenyl ring in a pocket which offers possibilities for more polar interactions than the pockets for the iodines of the inner ring.[30] The similarity of the arrangements of the L-T_4 diphenyl ether part, the dihydropyridines, and 2′,6′-dichloro-*N*-phenylanthranilic acid in their TTR complexes is visualized in Figure 5, which is obtained by fitting the protein backbones of the three optimized complexes and extracting the ligands in their optimum complex geometry. The dotted van der Waals surface areas illustrate the similarity of the size of the molecules.

On the basis of the optimized complexes, a survey of the hydrophobic and hydrogen-bonding capacities between TTR and the various ligands can be obtained by employing the program LIGPLOT,[45] which generates a 2D representation of a protein−ligand complex and provides information on hydrogen bonds and hydrophobic interactions which are estimated by consideration of characteristic structure criteria. Figure 6 provides this information for the TTR complexes with L-T_4, the very potent 3′- and 4′-nitro-substituted dihydropyridines, and the 2′,6′-dichloro-*N*-phenylanthranilic acid. For L-T_4, LIGPLOT shows only hydrophobic interactions (Figure 6a). It should be stressed that, beside hydrophobic interactions, only hydrogen bonds are predictable by LIGPLOT. Thus, other important interactions, *e.g.* between ionic groups, could be missing and the LIGPLOT information may be uncomplete. For instance,

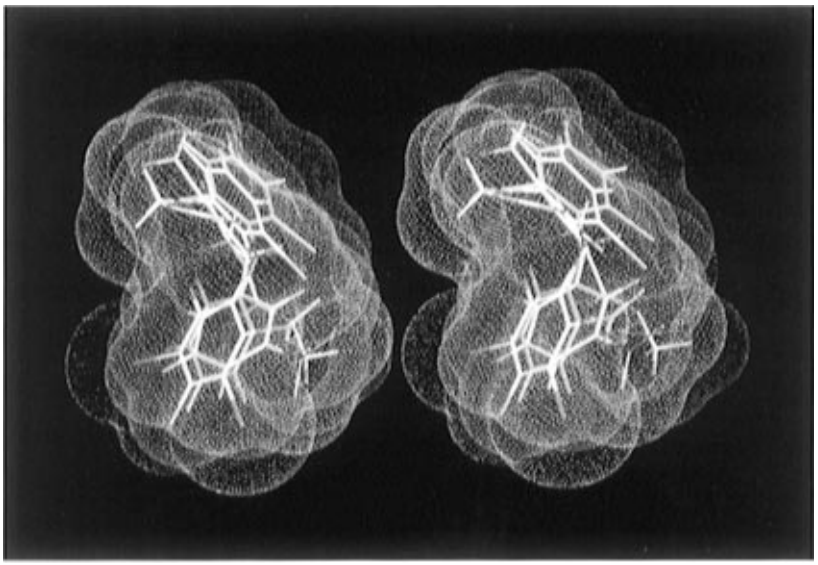


Figure 5. Stereoview of the superimposition of the L-T_4 diphenyl ether part (light rose) with the calcium antagonist 2,6-dimethyl-4-(3′-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid methyl ester (yellow), and 2′,6′-dichloro-*N*-phenylanthranilic acid (blue) in their optimum TTR complex arrangements extracted after fitting of the protein backbones (molecular van der Waals surface areas dotted).

in the case of L-T_4, the ionic contacts between Lys15 and Glu54 of TTR with the carboxy and ammonium groups of the L-T_4 alanine side chain are not indicated although the corresponding groups are in the correct interaction distance. Considerable hydrophobicity is also indicated for the other TTR−ligand complexes (Figures 6b−d). However, the two nitro derivatives show additional hydrogen bonds to amino acids of TTR which could be responsible for an increase of binding affinity and explain the high inhibitory potency of these compounds. Experimental studies suggest that the 4′-hydroxy group on the outer ring of the thyroid hormones is essential for binding and nearly completely ionized at physiological pH, *i.e.* binding is probably through a 4′-phenoxide ion.[21,22] It could be possible that the most potent 4′-nitrodihydropyridine mimics the 4′-phenoxide ion by the negative partial charges at the oxygen atoms of the nitro group. A hydrogen bond is also indicated between the carboxy group of the *N*-phenylanthranilic acid derivative and the protein.

In order to get more quantitative information, the stabilization energies for some representative TTR complexes with dihydropyridine derivatives were estimated in relation to the complexes of L-T_4 and its diphenyl ether part. At first, the influence of the L-Ala residue on the affinity was estimated by comparison of the TTR complexes with L-T_4 and only its diphenyl ether part, respectively. There is a stability difference of 42.7 kJ·mol^{-1} in favor of the L-T_4 complex. This value can fairly be compared with data from other authors[31] who found 29.3 kJ·mol^{-1} when considering a dielectric constant of $\epsilon = 2$, whereas the permittivity was $\epsilon = 5$ in our calculations. On comparison of the complexes with the 3′-nitro- and 3′-iodo-substituted dihydropyridine derivatives in their distal and proximal orientations, the distal conformers form distinctly more stable complexes. The stability differences between the two orientations are 20.5 kJ·mol^{-1} for the 3′-nitro and 29.2 kJ·mol^{-1} for the 3′-iodo derivatives. Experimental data for thyroid hormone derivatives with the distal and proximal orientations of the outer ring kept fixed show in fact that the distal conformers are more active.[22] The complex of the very potent 3′-nitrodihydropyridine derivative is by 31.4 kJ·mol^{-1} more stable than the 3′-iodinedihydropyridine complex, but it is still by 20.5 kJ·mol^{-1} more unstable than the TTR complex with the diphenyl ether part of L-T_4.

It has to be mentioned that two very important X-ray studies for TTR complexes with 3,3′-diiodo-L-thyronine (3,3′-T_2)[46] and 3′,5′-dinitro-*N*-acetyl-L-thyronine (DNNAT),[47] respectively, were recently published showing orientations of the ligands different from that found for L-T_4 in its complex. However, the missing of the second *ortho* iodine substituent in the inner ring of 3,3′-T_2 and the complete absence of both *ortho* substituents in DNNAT led to propeller-like minimum conformations different from the skewed conformation preferred in L-T_4 or L-T_3 and higher conformational flexibility as indicated by the results of molecular mechanics calculations. Thus, these ligands may realize different orientations in their TTR complexes. The X-ray data for these two complexes underline the importance of space-filling substituents at the *ortho* positions of the inner ring in the thyroid hormones for the realization of the skewed conformation. For the 4-phenyldihydropyridines, where the skewed form is anyway preferred and the ester groups may contribute to a stronger fixation of this conformation, and for the most active *N*-phenyl-anthranilic acid derivatives, where *ortho* chloro substituents could enforce a skewed-like arrangement additionally supported by the hydrogen bond between the carboxy group and the NH linkage, there may be some justification to assume an arrangement of the ligands which is comparable with L-T_4.

Conclusions

In our experimental L-T_3 uptake inhibition studies, some substituted dihydropyridine derivatives, which are also active as calcium antagonists, proved to be potent inhibitors. On the basis of the comparison between the measured inhibitory strengths and various structural properties additionally considering data from our former study on a series of *N*-phenylanthranilic acids, we postulate an angular orientation of the two

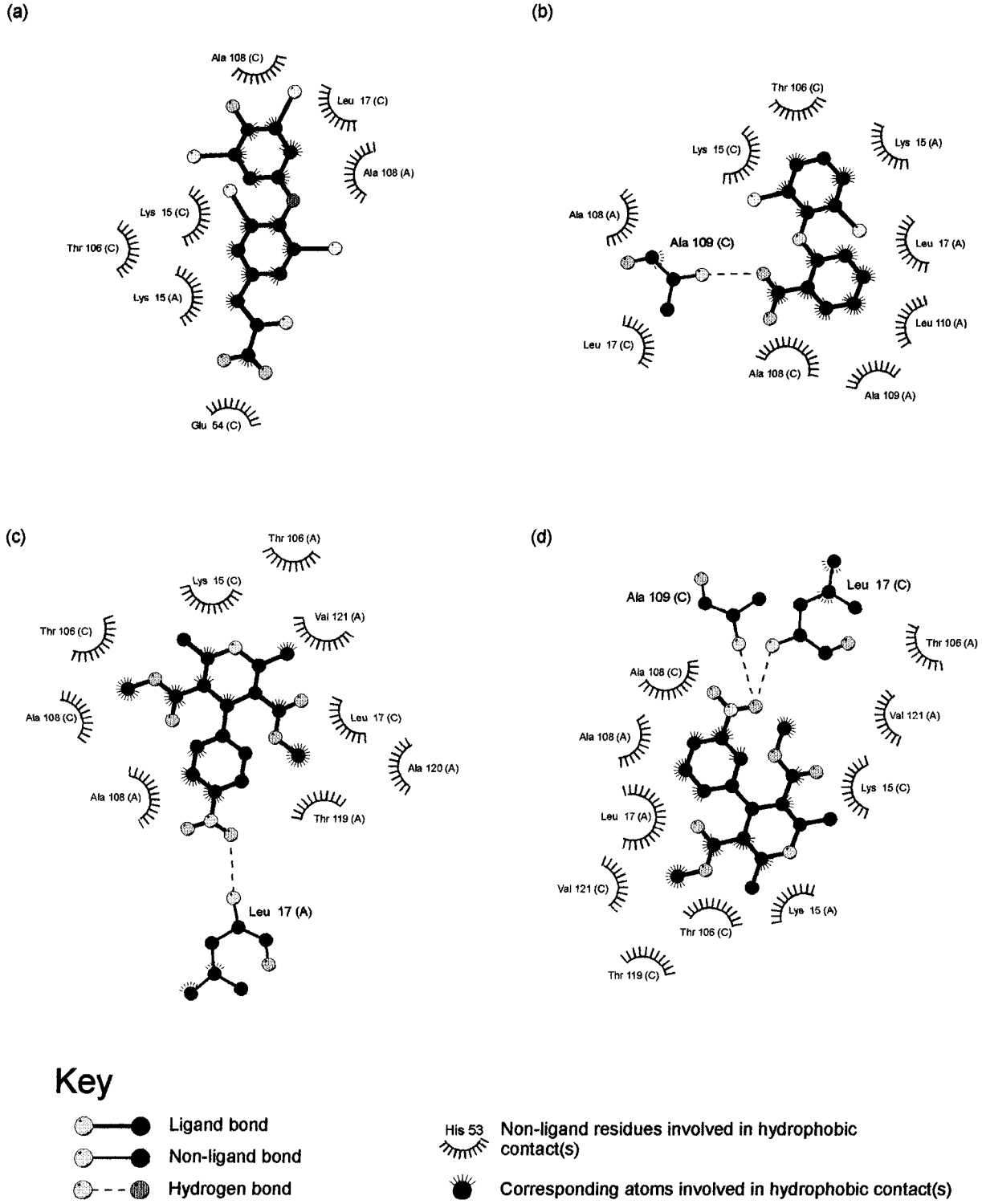


Figure 6. LIGPLOT representations of hydrophobic and hydrogen bond interactions in TTR complexes with (a) L-thyroxine, (b) 2′,6′-dichloro-*N*-phenylanthranilic acid, (c) 2,6-dimethyl-4-(4′-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid methyl ester, and (d) 2,6-dimethyl-4-(3′-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid methyl ester.

rings in these compounds as a prerequisite for inhibitory potency. The two rings have to be in a skewed or approximately skewed conformation, where the phenyl ring of the calcium antagonists is bisecting the dihydropyridine ring corresponding to the conformation of the outer phenyl ring of the thyroid hormones. In the *N*-phenylanthranilic acids, the phenyl ring bearing the carboxy group realizes a similar conformation. However, while lack of such an arrangement should be responsible for a loss of activity, the inhibition potency is also influenced by other factors such as the type of substituents influencing the interaction properties. For high inhibitory potency of 4-phenyldihydropyridines, nitro groups on the phenyl ring and ester groups with short alkyl chains on the dihydropyridine ring should be advantageous. Consideration of these aspects may eventually be useful for the development of efficient L-T_3 uptake inhibitors in the therapy of hyperthyroidism.

Experimental Section

Materials. Tissue culture media and supplements were from Gibco BRL (Eggenstein, Germany). Our source for ^{125}I-

L-T_3 was Brahms Diagnostica GmbH (Berlin, Germany). The unlabeled L-T_3 was purchased from Sigma (Deisenhofen, Germany). All dihydropyridine derivatives were supplied by the Bayer AG (Leverkusen, Germany). The human HepG2 (ATCC CRL 8065-HB) and H4 rat hepatoma cells (ATCC CRL 1548) came from the American Type Culture Collection (Rockville, MD).

L-T_3 Uptake. The screening procedure for the inhibitors of ^{125}I-L-T_3 uptake corresponds to Chalmers *et al.*[15] The cells were maintained in DMEM (Dulbecco's Modification of Eagle's Medium) with 10% fetal calf serum at 37 °C, subcultured for the uptake experiments in 2ml wells and grown to confluence (2×10^6 cells/well). Cultivation was continued in serum-free DMEM overnight. This medium was then replaced by DMEM containing 10^{-11} M ^{125}I-L-T_3 (specific activity >3200 μCi/μg). The uptake of ^{125}I-L-T_3 was measured after 2 min at 37°C. The nondisplaceable uptake was determined from duplicate incubations containing 10^{-5} M unlabelled L-T_3. For substance screening the test substances were included in a dose of 10^{-5} M together with ^{125}I-L-T_3. The IC_{50} determinations were performed in a dose range from 10^{-5} M to 10^{-9} M. The substances were dissolved in 100% DMSO for stock solutions of 10^{-2} M and added to DMEM to give the desired final concentration. The controls contained equivalent amounts of solvent. The uptake was terminated after incubation by decanting the medium and washing the cells five times with PBS (2 mL; pH 7.4) at room temperature. The cells were harvested with 2 mL of 0.1 M NaOH, and the uptake was measured as the cell-associated radioactivity.

Partition Coefficients. The log *P* values were determined by RP-HPLC using a RP C18 column (Tessek SeparonTM SGX C18). The elution of the substances was performed by methanol/water, 60:40 (v/v), with a flow rate of 0.8 mL·min^{-1} at 37 °C. In the case of basic compounds, triethylamine (25 mM) was added. The wavelength of UV detection was 230 nm. For a calibration curve the following substances were chromatographed as standards: acetophenone, acridine, anisol, benzene, benzophenone, quinoline, and naphthalene. The calculation of the 1-octanol/water partition coefficients is based on the program PALLAS, version 1.1.[43]

Calculations. The molecular mechanics and dynamics calculations were performed on the basis of the modeling software QUANTA, version 4.1,[48] employing the CHARMm 23.1 force field which provides parameters for all structures. The charges were calculated according to Gasteiger and Marsili.[49] The rotation angle step size was 10° in the conformational searches from which the minimum conformations were extracted and then reoptimized. In the optimizations of the TTR−ligand complexes, the united atom CHARMm force field was employed for the protein part, whereas the all-atom force field was used for the ligands. In order to avoid an overestimation of the influence of electrostatic interactions in the protein, a dielectric constant of $\epsilon = 5$ was applied. The transthyretin is a tetramer consisting of four identical subunits which are oriented to form a central channel containing two L-T_4 binding sites.[30,44] For our purposes a dimer unit with one L-T_4 molecule was selected. The starting structures for the optmization of the TTR-dihydropyridine complexes were generated by fitting the dihydropyridine ring of the optimized derivatives onto the inner ring of the L-T_4 diphenyl ether moiety in the X-ray structure of the TTR−L-T_4 complex as perfectly as possible by rigid body fit.

For the estimation of the hydrophobic and hydrogen bond interactions in the protein−ligand complexes the program LIGPLOT, version 2.0, was used.[45] The molecular volumes and the electrostatic potentials of the ligand molecules were estimated by means of the program GRASP, version 1.3.6.[50]

The molecular dynamics trajectories for the determination of the conformational flexibilities of the ligands are based on 100 ps simulations after heating from 0 to 300 K and equilibration within 20 ps. The integration step size was 1 fs. The time of 100 ps is completely sufficient to determine the range of variation of the torsion angles in all derivatives.

The quantum chemical geometry optimizations were performed at the HF/6-31G* level of *ab initio* MO theory[51] employing the program SPARTAN, versions 3.1 and 4.1.[52]

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