Biochemical Pharmacology, Vol. 32, No. 14, pp. 2175–2180, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

MODE OF INSERTION OF MICONAZOLE, KETOCONAZOLE AND DEACYLATED KETOCONAZOLE IN LIPID LAYERS

A CONFORMATIONAL ANALYSIS

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(*Received* 16 *November* 1982; *accepted* 7 *February* 1983)

Abstract—The conformation of three imidazole derivatives, miconazole, ketoconazole and deacylated ketoconazole (R 39 519) inserted in a lipid layer was calculated using a procedure of conformational analysis. For each imidazole derivative all probable conformers were inserted into a dipalmitoyl phosphatidylcholine (DPPC) monolayer. Miconazole maintains its two dichlorophenyl groups in the hydrophobic phase whereas the imidazole moiety is orientated in the hydrophilic phase. Ketoconazole orientates its dichlorophenyl group in the hydrophobic phase whereas its acylated piperazine moiety is orientated towards the hydrophobic region. Deacylation inverses completely the orientation of the compound. The most probable conformer of R 39 519 is inserted in the lipid layer with its piperazine moiety orientated towards the aqueous phase. The inversion increases the area occupied per drug molecule from 30 Å^2 for ketoconazole to 90 Å^2 for R 39 519 equal to the mean area occupied per miconazole molecule and higher than that occupied per DPPC molecule (60 Å^2). Such a conformation should result in a destabilizing effect of miconazole and R 39 519; this was proved using differential scanning calorimetry.

Miconazole† and ketoconazole‡ are imidazole derivatives with broad spectrum activity against yeast and fungi [1, 2]. *In vitro*, miconazole is also active against Gram-positive bacteria [1, 3], an activity it shares with ketoconazole. However, as compared with miconazole, almost 16 times more ketoconazole is needed [4]. At high doses both antifungal compounds are active *in vitro* against *Leishmania tropica* [5] and some activity was also found against *Plasmodium falciparum* [6]. It is of interest that, compared with ketoconazole, deacylated ketoconazole§ (R 39 519) has *in vitro* higher anti-leishmanial [5] and anti-malarial [6] activities.

Both miconazole [7] and ketoconazole [8] affect ergosterol synthesis in yeast cells, resulting in an accumulation of 14α-methylsterols, and induce a shift from unsaturated (18:1) to saturated and shorter (16:0) fatty acids [4, 9]. The latter effect enhances the membrane disturbances induced by the accumulation of the 14α-methylsterols, decreases growth, and leads to decreased activity of membrane-bound enzymes.

Recent studies show that miconazole interferes with a third target. Using differential scanning calorimetry (DSC) it was shown that high doses of miconazole shift the lipid transition temperature of multilamellar vesicles to lower values without affecting the enthalpy of melting [4]. In the presence of ketoconazole no significant shift of the main dipalmitoyl phosphatidylcholine (DPPC) transition temperature was observed [4].

The miconazole-induced shift of the lipid transition temperature to lower values is suggestive of a change in lipid organization. In this study the different mode of organization is further demonstrated by a new procedure of conformational analysis [10, 11]. The latter method is also used to describe the orientation of deacylated ketoconazole, the DSC curves of which show a similar shift of the main DPPC transition temperature as that observed with miconazole.

MATERIALS AND METHODS

Differential scanning calorimetry studies. Multilamellar vesicles of dipalmitoyl phosphatidylcholine (DPPC, Sigma) were prepared at a lipid concentration of 55 μmole/ml in Tris–HCl buffer (10^{-2} M, pH 7.3, containing 0.15 M NaCl) as previously described [12]. Deacylated ketoconazole (R 39 519, Janssen Pharmaceutica) was incorporated in the lipid film prior to the liposome formation.

DSC measurements were carried out as previously dessribed [4].

Conformation and orientation of isolated molecules. The method used for the conformational analysis of each drug (miconazole, ketoconazole and deacylated ketoconazole) is based on a strategy

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† 1 - [2 - (2,4 - Dichlorophenyl) - 2 - [(2,4 - dichlorophenyl)methoxy]ethyl] - 1H - imidazole mononitrate.

‡ Cis - 1 - acetyl - 4 - {4 - [2 - (2,4 - dichlorophenyl) - 2 (1H - imidazol - 1 - yl - methyl) - 1,3 - dioxolan - 4 - yl]-methoxy]phenyl}piperazine.

§ Cis - 1 - {4 - [2 - (2,4 - dichlorophenyl) - 2(1H - imidazol-1-ylmethyl) - 1,3 - dioxolan - 4 - ylmethoxy]phenyl}piperazine.

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described elsewhere [10, 11] and currently used for studying the conformation of polypeptides [13, 14] and other molecules [15, 16].

In this method, the total conformational energy is empirically calculated as the sum of the contributions resulting from the Van der Waals interactions, the torsional potentials and the electrostatic interactions. The latter was calculated for a dielectric constant of 16, a value intermediate to that currently used for the aqueous and hydrophobic phase at a simulated interface [11]. The values used for valence angles, bond lengths, atomic charges and torsional potentials are those used in conformational analysis [17].

In a first systematic study, the torsional angles of each drug underwent successive increments of 60° each, yielding 6^n different conformations derived from the all *trans* conformer, arbitrarily taken as the initial conformation.

A torsional angle around a given j bond is taken as positive when the distal $(j+1)$ bond rotates clockwise relative to the proximal $(j-1)$ bond [18]. The conformations obtained from this first study and yielding a low internal energy, i.e. those with a statistical weight of at least 1%, were then submitted to a simplex minimization procedure [19].

In the last step of the analytical procedure, the hydrophobic and hydrophilic gravity centres of selected conformers were established taking into account the transfer energy [20] of each part of the molecule (Fig. 1).

The hydrophilic gravity centre (C_{tr}^{phi}) is defined by the following equation

$$C_{tr}^{phi} = \sum_{i=1}^{n} [E_{tr_i}^{phi} \cdot \vec{r}_i] / \sum_{i=1}^{n} E_{tr_i}^{phi}$$

in which $\vec{r}_i$ are the coordinates of the ith atom. The hydrophobic gravity centre located in the hydrocarbon domain C_{tr}^{pho} is defined by the same equation, except that the negative transfer energy is taken into account [20].

The values for the transfer energy used here were determined experimentally by numerous authors and summarized elsewhere [20]. The interface position (I) is defined by the equation

$$\frac{\sum_{i=1}^{n} E_{tr_i}^{phi}}{|(C_{tr}^{phi} - I)|} = \frac{\sum_{j=1}^{m} E_{tr_j}^{pho}}{|(C_{tr}^{pho} - I)|}$$

In the second step of the procedure, the assembly of molecules in the monolayer was computed as follows.

Conformation of the drug molecule inserted into the lipid monolayer. The procedure of drug insertion can be summarized as follows: (a) the position of drug A (orientation of the isolated molecule) was modified along the X-axis. Each distance was equal to 0.5 Å. For each separating distance a rotation angle of 30° was imposed to drug A around its own Z-axis and around the lipid (B) (Fig. 2, a and b). Among 14,400 possible orientations only the structure of minimum energy was considered. (b) Lipid B was fixed and drug A was allowed to move along the Z-axis perpendicular to the lipid–water interface (Fig. 2c). Again only the structure of minimum energy was considered. (c) Drug A had the possibility of changing its orientation around the Z-axis compared with lipid B (Fig. 2c). This procedure allowed the probable packing of drug and lipid molecules to be defined. Then the packing of these two molecules was maintained and the orientation of a third lipid

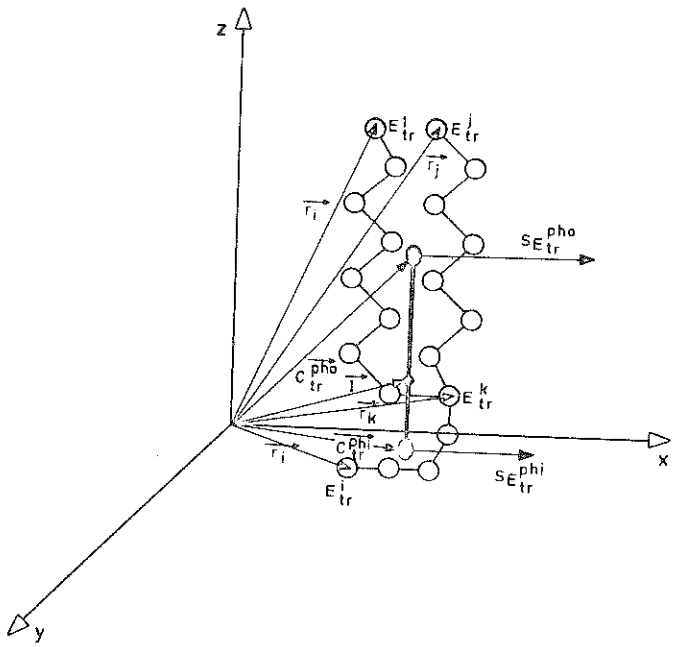

Fig. 1. Definition of the coordinates (X, Y, Z) of the hydrophobic gravity centre, hydrophilic gravity centre and the interface, with: $E_{tr}^{i,\ldots}$ the transfer energy of the atoms $1, \ldots, j, k, i$; $r_1, \ldots,$ the coordinates of the atom $1, \ldots, j, k, i$; C_{tr}^{pho} the coordinates of the hydrophobic gravity centre (see text); C_{tr}^{phi} the coordinates of the hydrophilic gravity centre (see text); SE_{tr}^{phi}, SE_{tr}^{phi} the sum of the hydrophobic transfer energy and the sum of the hydrophilic transfer energy, respectively. I the coordinates of the interface (see text).

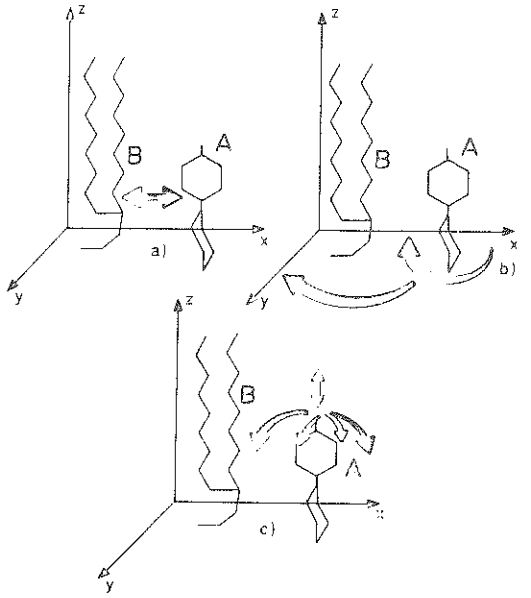

Fig. 2. Schematic presentation of the packing procedure of molecules assembled in mixed monolayer

molecule around them was considered. Because this was time-consuming, we limited our approach to the number of lipid molecules sufficient to surround the drug.

When the configuration of the cluster of m molecules was determined, both areas occupied by each molecule and the intermolecular area were estimated after projection on the x–y plane, and the mean molecular area was calculated.

RESULTS

The conformation of three imidazole derivatives inserted into a lipid layer was calculated using the procedure described in Materials and Methods.

Conformation systematic study

Initial systematic study. Miconazole: The torsional angles (θ_1, θ_2, θ_3, θ_4, θ_5, θ_6) of miconazole (Fig. 3a) were given successive increments of 60°, yielding 46,656 different conformations from which two structures with maximal probability were retained. All other conformers presented a probability of existence below 1% (Table 1).

Ketoconazole: The torsional angles (θ_1, θ_2, θ_3, α_1, α_2, α_3, α_4, α_5) of the ketoconazole molecule (Fig. 3b) were increased by steps of 60°, yielding 279,936 conformations from which three structures were selected (Table 1).

Deacylated ketoconazole (R 39 519): The systematic study was performed on the same angles as for ketoconazole (Fig. 3c). Again only the three most probable conformers were retained (Table 1).

Minimization procedure and interfacial orientation. The values of the torsional angles obtained after application of the simplex minimization procedure and orientation of the molecule at the simulated membrane–water interface are listed in Table 2, which includes the distance between the hydrophobic and hydrophilic centres of each conformer

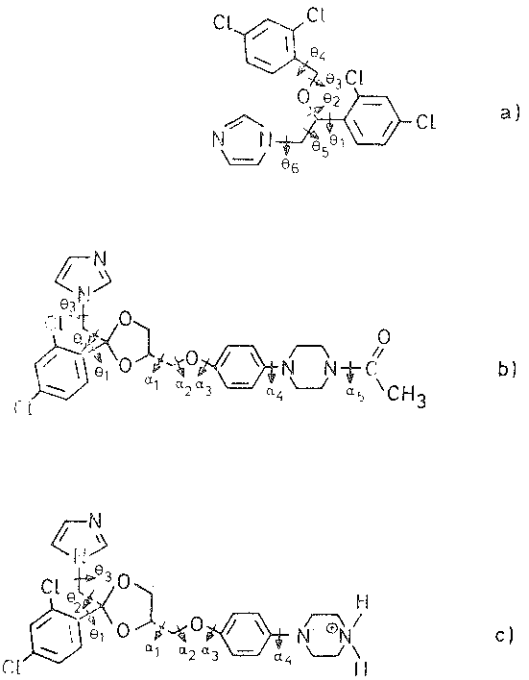

Fig. 3. Definition of the torsional angles in imidazole derivatives: (a) miconazole; (b) ketoconazole; and (c) deacylated ketoconazole.

and also the energy (hydrophobic and hydrophilic) associated with these two centres.

Conformation of the drug molecule inserted into the lipid monolayers

For each imidazole derivative, all probable conformers were inserted in a DPPC monolayer but only the assembling modes corresponding to the minimal energy were retained. The structures shown in Figs. 4, 5 and 6 correspond to a probability of 99%.

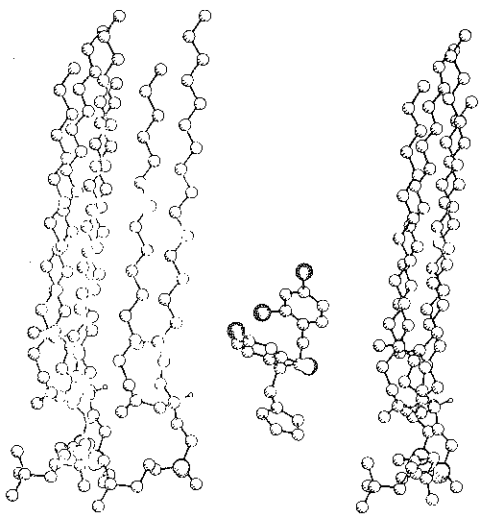

Fig. 4. Configuration of miconazole–DPPC mixed monolayer (lipids placed in front and behind the miconazole are not represented). Open circles refer to carbon atoms; filled circles refer to Cl atoms; O symbol represents a P atom.

Table 1. Most probable conformers of the imidazole derivatives

(a) Miconazole

	θ_1	θ_2	θ_3	θ_4	θ_5	θ_6	Probability (%)	Energy above minimal value (kcal/mole)
A	240	240	300	120	180	240	9.95	0
B	240	240	300	120	0	240	9.85	0.07

(b) Ketoconazole

	θ_1	θ_2	θ_3	α_1	α_2	α_3	α_4	α_5	Probability (%)	Energy above minimal value (kcal/mole)
A	0	180	240	0	180	120	180	180	3.58	0
B	0	180	120	0	180	120	180	180	3.53	0.01
C	0	180	120	0	180	300	180	180	3.51	0.01

(c)Deacylated ketoconazole

	θ_1	θ_2	θ_3	α_1	α_2	α_3	α_4	Probability (%)	Energy above minimal value (kcal/mole)
A	0	180	240	0	180	300	180	3.26	0
B	0	180	120	0	180	300	180	3.10	0.03
C	0	180	120	0	180	120	180	3.02	0.06

Table 2. Most probable conformers after minimization and orientation at the lipid–water interface

(a) Miconazole

	θ_1	θ_2	θ_3	θ_4	θ_5	θ_6	Distance between hydrophilic and hydrophobic centres (Å)	Hydrophobic transfer energy (kcal/mole)	Hydrophilic transfer energy (kcal/mole)
A	243	229	303	105	196	270	3.1	32	9
B	237	238	320	102	16	276	3.3		

(b) Ketoconazole

	θ_1	θ_2	θ_3	α_1	α_2	α_3	α_4	α_5	Distance between hydrophilic and hydrophobic centres (Å)	Hydrophobic transfer energy (kcal/mole)	Hydrophilic transfer energy (kcal/mole)
A	348	182	283	1	174	95	89	247	2.1	48	23
B	348	182	258	1	174	81	90	117	2.1		
C	347	181	283	344	11	334	88	234	1.3		

(c) Deacylated ketoconazole

	θ_1	θ_2	θ_3	α_1	α_2	α_3	α_4	Distance between hydrophilic and hydrophobic centres (Å)	Hydrophobic transfer energy (kcal/mole)	Hydrophilic transfer energy (kcal/mole)
A	346	184	284	4	147	263	89	0.8	44	42
B	345	183	103	3	170	271	87	0.8		
C	348	186	99	0	168	85	94	0.5		

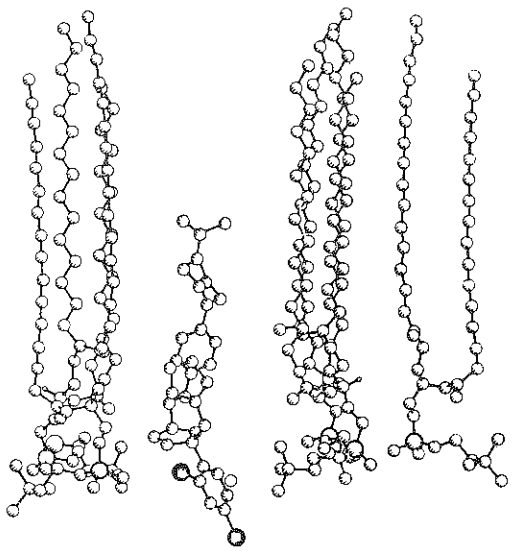

Fig. 5. Configuration of ketoconazole–DPPC mixed monolayer (lipids placed in front of and behind the ketoconazole are not represented). Symbols for the various atoms are the same as in Fig. 4.

Miconazole. Each miconazole molecule is surrounded by seven lipid molecules (lipids placed in front and behind the miconazole were not represented). Miconazole (Fig. 4) maintains its two dichlorophenyl groups in the hydrophobic phase; the imidazole moiety is orientated in the hydrophilic phase.

The mean area occupied per drug molecule (90 Å^2) differs from the mean area occupied per lipid (DPPC 60 Å^2). Such a conformation is supposed to modify the lipid layer organization and is in agreement with experimental facts. Indeed, as shown previously, miconazole strongly modifies the differential scanning calorimetry spectra of DPPC [4]; the peak characterizing the DPPC phase is strongly shifted to lower temperatures [4]. Maximal shift is observed for a drug molar concentration between 10 and 20%. This concentration corresponds to the number of lipid molecules needed to surround one drug molecule.

The fact that miconazole does not significantly affect the DPPC enthalpy of melting [4] suggests that the drug enhances the distance between adjacent lipids but does not affect the lipid conformation. This fact was confirmed in our theoretical approach by the non-modification of the lipid orientation. Indeed, no change in the lipid molecular organization was observed in the presence or absence of miconazole (Fig. 4).

Ketoconazole. The area occupied per drug molecule (30 Å^2) is much lower than for miconazole. Inserted into the lipid layer, ketoconazole orientates its piperazine moiety towards the hydrophobic region and the dichlorophenyl group in the hydrophilic phase (Fig. 5). Such an orientation is not supposed to affect the lipid organization. Indeed, DSC measurements have shown that even at high drug concentrations (30 mole %) ketoconazole does not induce significant changes of the DPPC transition temperature and of the lipid enthalpy of melting [4].

Deacylated ketoconazole. Deacylation of ketoconazole causes a drastic conformational change. Indeed, the most probable conformer (Fig. 6) is inserted in the lipid layer with its piperazine moiety orientated towards the aqueous phase. This inversion is the consequence of the proximity of the hydrophobic and hydrophilic gravity centres which reduces the energy necessary to allow the flip-flop motion of the drug. A direct consequence of this inversion is the increase in the area occupied per drug molecule (90 Å^2) in the lipid layer. The DSC spectra (Fig. 7) confirmed the expected destabilizing effect; a shift of the mean DPPC transition was observed and was

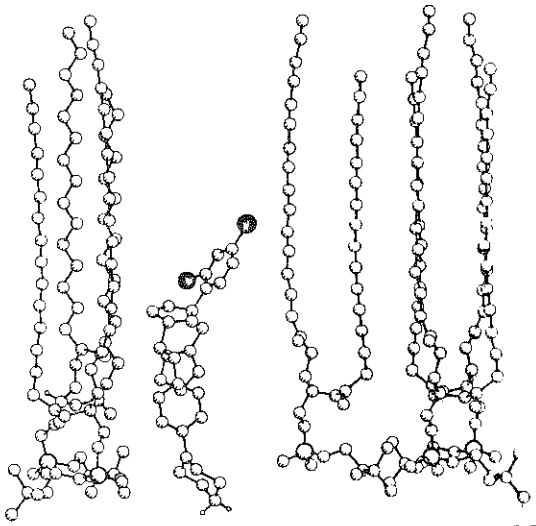

Fig. 6. Configuration of deacylated ketoconazole–DPPC mixed monolayer (lipids placed in front of and behind the ketoconazole are not represented). Symbols for the various atoms are the same as in Fig. 4.

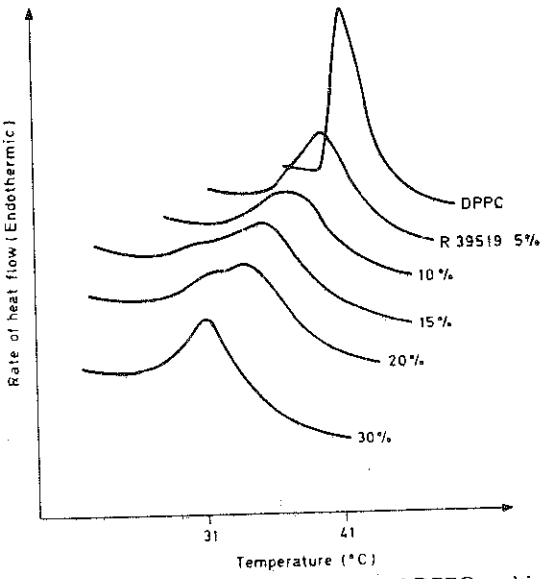

Fig. 7. Differential scanning calorimetry of DPPC multiamellar vesicles containing increasing molar amounts of deacylated ketoconazole (R 39 519). Lipid concentration: 55 μmole/ml. Liposomes were formed in Tris-HCl buffer (10 mM, pH 7.3, 0.15 M NaCl). Drug–lipid molar ratios are indicated in the figure.

Table 3. Enthalpy of melting*

Liposomes	Molar ratio	Δ H (main transition) (kcal/mole)
DPPC/DPPC	100/0	8.0
DPPC/R 39 519	100/2.5	7.5
	100/10	7.2
	100/15	7.7
	100/20	8.0
	100/30	8.0

* Lipid concentrations: 55 μmole/ml. Liposomes were formed in Tris–HCl buffer (10^{-2} M, pH 7.3, 0.15 M NaCl). R 39 519 = deacylated ketoconazole.

maximal for 30 mole % of drug. The non-modification of the DPPC enthalpy of melting (Table 3) even at high drug concentrations suggests that the lipid orientation is not affected by the drug, which acts as a spacer between the lipid acyl chains.

DISCUSSION

The new conformational analysis presented here allows a molecular description of the drug position when inserted in the lipid layer. The proposed orientations are in agreement with the experimental observations. This calculation is possible for any drug where the number of atoms does not exceed 100.

The interesting points are that screening can be done for a great number of imidazole derivatives, and that conformational parameters essential to induce a lipid destabilization can be defined before any synthesis. An important point is the difference observed between ketoconazole and deacylated ketoconazole. It shows that the vicinity of hydrophobic and hydrophilic centres of gravity is favourable for a possible reorientation in the lipid layer. Clearly, a minor modification of a residue in the drug structure can drastically enhance the drug capacity to destabilize the membrane structure.

The good correlation already obtained between experimental data and our theoretical predictions for a lipidic system [10] suggests that the computational approach could be a valuable tool in obtaining a clear picture of the orientation of any drug in the lipid bilayer of the membrane. This procedure should be completed with a basic experimental approach, such as neutron diffraction [21].

Acknowledgements—Appreciation is expressed to H. Vanhove for help in preparing the manuscript and to D. Verkuringen for typing it.

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