Biochemical Pharmacology, Vol. 34, No. 7, pp. 1073–1077, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

STRUCTURE–ACTIVITY RELATIONSHIPS OF BENZIMIDAZOLE CARBAMATES AS INHIBITORS OF MAMMALIAN TUBULIN, *IN VITRO*

ERNEST LACEY* and THOMAS R. WATSON
Pharmacy Department, Sydney University, Sydney N.S.W. 2006, Australia

(*Received* 29 *February* 1984; *accepted* 25 *July* 1984)

Abstract—The structure–activity relationships of thirty-two methyl (5(6)-substituted benzimidazol-2yl) carbamates as inhibitors of the rate of polymerisation of mammalian tubulin have been investigated. The size or some colinear physico-chemical characteristic of the substituent in the 5 (or 6)-position has a profound effect on potency. The presence of branching with or without a commensurate increase in the polarity of the 5(6)-substituent adjacent to the benzimidazole ring (α-position) resulted in a loss of activity. The nature of the overall site, as reflected by the quantitative models, could relate to either the hydrophobicity or molar volume of the 5 (or 6)-substituents.

The polymerisation of tubulin to form microtubules is an essential function in a wide variety of structural, mitotic, secretory and transport phenomena in eukaryotic cells [1, 2]. Inhibitors of this polymerisation, such as colchicine, vinblastine, maytansine and their analogues, have been shown to exhibit experimentally, and subsequently clinically, useful antitumour activity as a consequence of selective toxicity for the mitosis of rapidly dividing tumour cells [3–6]. Benzimidazole carbamates (Fig. 1, IV), a class of broad spectrum antifungal [7] and anthelmintic [8–13] compounds, have also been shown to inhibit tubulin polymerisation by competing for the colchicine binding site on tubulin [14–16]. These observations have led to the hypothesis that tubulin is the primary target of this class in fungal and helminth species.

To date, investigation of the structure–activity relationships of benzimidazole carbamates against mammalian tubulin has been limited to commercially available anthelmintics [14–16]. Although these studies highlighted the importance of the substituent in the 5(6)-position of the benzimidazole ring, they enable only limited empirical comparison of a number of closely related compounds. To more fully characterise the role of the 5(6)-position, this study was undertaken to generate quantitative models for the relationship between inhibition of tubulin polymerisation and substituent structure. Using these models, it will be possible to examine potential lines of synthetic development of superior inhibitors of tubulin polymerisation and subsequently potential anti-tumour agents. The development of such models will also enable a greater understanding of the selectivity of action of particular derivatives as either antitumour, antifungal or anthelmintic agents by comparison with models obtained with helminth and fungal screens.

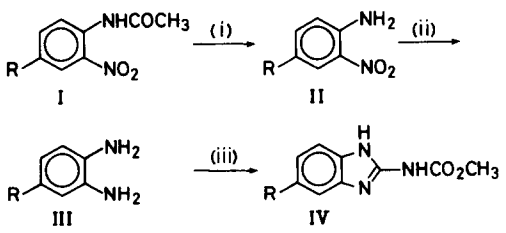

Fig. 1. Method of synthesis of methyl (5(6)-substituted benzimidazole-2yl) carbamates.

MATERIALS AND METHODS

The methyl (5(6)-substituted benzimidazol-2yl) carbamates (IV) (Table 1, compounds IV, 1–3, 5–10, 12–22, 24, 25) were synthesised by the cyclisation of the appropriate 1,2-diaminobenzene (III) with 1,3-*bis*-methoxycarbonylisothiourea as outlined in Fig. 1 [13].

The 4-substituted-1,2-diaminobenzenes (III) were prepared by catalytic reduction of the 2-nitroanilines (II) using either palladium on charcoal or Raney nickel as catalyst. In most of these syntheses, the intermediate diamine (III) was not isolated but, after normal work-up, was reacted immediately to form the benzimidazole carbamate (IV). However, under these conditions, catalytic debromination of intermediate (II) in the synthesis of (IV, 6) occurred. Such halogenated intermediates were reduced with dithionite to avoid dehalogenation.

The 4-alkyl-2-nitroanilines (II) were prepared by acetylation, nitration and saponification of the appropriate 4-alkylaniline using the method of Di Cuollo *et al.* [8]. The 4-alkoxy-2-nitroanilines were prepared from 4-hydroxyacetanilide by reaction with the appropriate alkylhalide [17], nitration

* Present address: McMaster Laboratory, CSIRO Division of Animal Health, Private Bag No. 1, P.O., Glebe, N.S.W. 2037, Australia.

[18] and saponification [18]. Methyl (5(6)-phenoxybenzimidazol-2yl) carbamate (Table 1, IV, 25) was prepared from 5-chloro-2-nitroaniline [19] by nucleophilic substitution with phenol (an analogous reaction to that published by Averkin *et al.* [13]) and subsequent reactions as shown in Fig. 1.

The starting materials for the synthesis of (IV, 6) and (IV, 7) were prepared by the bromination and thiocyanation of 2-nitroaniline using published methods [20, 21].

Commercially available derivatives of either (II) or (III) were used to prepare compounds (IV, 1, 2, 5, 8, 9, 10 and 19) according to Fig. 1.

Compounds (IV, 4) and (IV, 29) were prepared by the reduction of (IV, 8) and (IV, 28) using alkaline dithionite and sodium borohydride respectively [22].

All benzimidazole carbamates were characterized by NMR, MS and elemental analysis. NMR spectra were obtained using a Joel FX90Q NMR spectrometer (90 MHz), using tetramethylsilane (TMS) as reference ($\delta = 0.00$ ppm) in either $CDCl_3$ or CD_3SOCD_3. Mass spectra were obtained using a Finnigan Quadrupole 3200 Gas Chromatograph/Mass Spectrometer interfaced with a 6110 Data System. Chemical ionisation was achieved using methane gas. Microanalyses were obtained from Microanalytical Service, Melbourne, Australia.

The microtubules used in this work were isolated from sheep brain according to the method of Shelanski *et al.* [23] with the following modifications.

After homogenisation, a pre-spin at 10,000 *g* for 30 min was introduced to remove heavy precipitates. The second polymerisation was carried out in the absence of glycerol, and microtubules precipitated after the second polymerisation were collected and frozen in liquid nitrogen until ready for use. Microtubules stored in this way were used within 10 days.

Prior to inhibition studies, microtubules frozen in liquid nitrogen were thawed to room temperature and suspended in 0.1 M morpholinoethane sulphonic acid buffer containing 1.0 mM GTP, 1.0 mM ethyleneglycolbis(amino-ethylether)tetra acetate (EGTA), and 0.05 M $MgCl_2$ at pH 6.7 and maintained at 4° for 30 min to allow depolymerisation to tubulin. The solution was then centrifuged at 100,000 *g* for 15 min at 4° to remove tubulin aggregates. The clear solution was diluted to a protein concentration of approximately 1.4 mg/ml. This concentration of tubulin gave an absorbance change of approximately 0.2 absorbance units on polymerisation to form microtubules.

For the inhibition studies, 0.005 ml of an inhibitor solution in dimethylsulfoxide (DMSO) was added to 0.5 ml of the tubulin solution in a polystyrene spectrophotometric cuvette (Solandra Scientific Co.). The solution was gently vortexed and incubated at 37°, for 15 min, and the absorbance change was monitored using a Beckman model 25 spectrophotometer (Fig. 2).

Generally, five to six concentrations of inhibitor were examined in duplicate to produce a range of inhibition between 10 and 90%. The rate of polymerisation for each concentration was obtained graphically from the linear portion of the polymerisation profile and was used to calculate a percentage inhibition of polymerization [24] on duplicate batches of tubulin. IC_{50} Values (that is, the concentration required to inhibit the rate of polymerisation by 50%) were calculated using the least squares fit programme on a Hewlett–Packard HP-33 calculator.

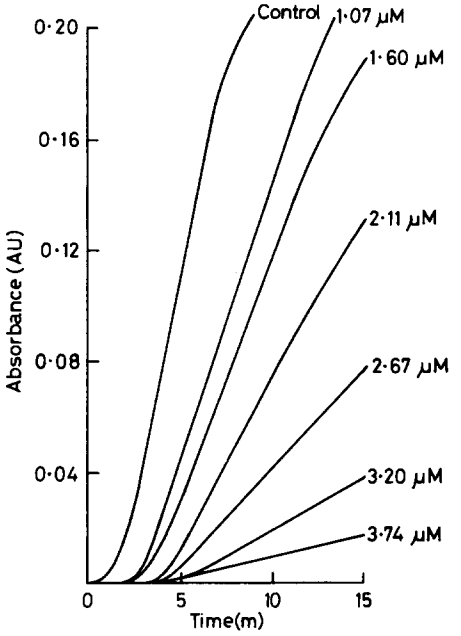

Fig. 2. Inhibition in the rate of polymerisation of mammalian tubulin by flubendazole (IV, 30).

For quantitative structure–activity analysis, the pIC_{50} values (negative logarithm of the IC_{50} value) were used.

The values for the physico-chemical parameters (MR, π and L) were obtained from the compilation of Hansch and Leo [25] and Verloop *et al.* [26]. The indicator variable I_{alkyl}, takes the value of 1 when the compound contains a $-CH_2CH_2R$ or $-OCH_2R$, where R is larger than H, joined to the benzimidazole nucleus. For all other substituents, $I_{alkyl} = 0$.

The analysis of the physico-chemical parameters as a function of IC_{50} was done using the "Funfit" interactive time-sharing programme as described by Pedersen [27] on a Cyber 72 computer (University of Sydney). The programme computed the curves of best fit for each group of compounds using the method of least squares. The quality of fit of the observed data to the calculated response curve was examined by the three statistics: (1) the correlation coefficient (r), (2) the percentage of data variance ($r^2 \times 100$) and (3) the standard deviation from the regression (S.D.).

RESULTS AND DISCUSSION

Figure 2 shows the inhibition of the rate of polymerisation of tubulin by flubendazole (IV, 30). This dose–response is typical of all of the benzimidazole carbamates that show tubulin polymerisation inhibitory activity. The delay in onset of polymerisation has been observed previously [24] and appears to be concentration dependent since the onset-delay increases with increasing inhibitor concentration.

Table 1. Inhibition of the rate of polymerisation of sheep brain tubulin by methyl (5(6)-substituted benzimidazole-2yl) carbamates

Compound No. (IV)	R*	IC_{50}† (μM)
1	H	71
2	F	82
3	OH	NI 126‡
4	NH_2	NI 213
5	Cl	18
6	Br	5.7
7	SCN	19
8	NO_2	NI 100
9	OCH_3	57
10	OCH_2CH_3	7.4
11	$OCH_2CH_2CH_3$§	2.4
12	$OCH(CH_3)_2$	7.4
13	*n*-OC_4H_9	3.3
14	*iso*-OC_4H_9	3.1
15	*n*-OC_5H_{11}	2.8
16	*n*-OC_6H_{13}	2.2
17	*n*-OC_7H_{15}	2.1
18	*n*-OC_8H_{17}	3.2
19	CH_3	16
20	CH_2CH_3	4.9
21	$CH_2CH_2CH_3$	2.2
22	$CH(CH_3)_2$	16
23	$CH_2CH_2CH_2CH_3$§	3.1
24	$C(CH_3)_3$	41
25	OC_6H_5	3.3
26	SC_6H_5‖	5.4
27	SOC_6H_5¶	NI 100
28	COC_6H_5**	6.1
29	$CHOHC_6H_5$	70
30	COC_6H_4F**	3.5
31	CO—C_4H_4S**	2.1
32	$SCH_2CH_2CH_3$§	6.9

* For complete structure, see Fig. 1.

† IC_{50} (μM) is the concentration in micromolar required to provide 50% inhibition of polymerisation.

‡ NI indicates no inhibition at the maximum concentration used (generally limited by solubility under assay conditions).

§ Oxibendazole, parbendazole and albendazole were gifts from Smith, Kline & French, Australia.

‖ Fenbendazole was a gift from Hoescht, Australia.

¶ Oxfendazole was a gift from Wellcome, Australia.

** Mebendazole, flubendazole and nocodazole were gifts from Janssen Pharmaceutica, Australia.

The relative potency of the compounds does not appear to affect the delay in the time of onset of polymerisation.

The concentration range required to achieve between 10 and 90% reduction in the rate of polymerisation compared with the control was narrow. Typically, a 4- to 5-fold increase in concentration was required. The computed regression line of the inhibition of the rate of polymerisation versus concentration was linear over the range of 10–90% inhibition. For all of the inhibitors tested, the correlation coefficient for the line of best fit was greater than 0.95.

The reproducibility of batches of tubulin was monitored by routinely testing the inhibitory activity of oxibendazole (IV, 11). Over fifteen batches, the IC_{50} of oxibendazole was found to be 2.44 ± 0.14 μM.

The IC_{50} values obtained in this study (Table 1) are in good agreement with those values previously reported for commercial anthelmintics using bovine brain tubulin [14] and sheep brain tubulin [15]. Methyl benzimidazol-2yl carbamate (IV, 1), the unsubstituted parent compound of this series of compounds, was found to be a weak inhibitor of the rate of polymerisation ($IC_{50} = 71$ μM). Replacement of the 5 (or 6) hydrogen with fluorine (IV, 2) gave a slightly less potent inhibitor ($IC_{50} = 82$ μM), whereas the introduction of larger halides, Cl (IV, 5) and Br (IV, 6), resulted in a progressive improvement in potency. The presence of polar groups, whether electron-withdrawing (NO_2, IV, 8) or electron-donating (OH, IV, 3; NH_2, IV, 4) resulted in a loss of activity.

The effect of increasing the size of the 5(6)-substituent was examined by measuring the activity of a series of compounds having straight chain alkoxy substituents (IV, 9–11, 13, 15–18). An increase in the alkoxy chain length from methoxy (IV, 9) to propoxy (IV, 11) resulted in a 23-fold increase in potency, but increasing the length further to octyloxy (IV, 18) resulted in little change from the propoxy in the IC_{50} value. A similar increase in potency was shown for the 5(6)-alkyl series (IV, 19–21, 23). These results show the importance of the size of the 5(6)-substituent on activity. Whether the size of the substituent *per se* is important or whether a physicochemical property which is colinear for this series of substituents is involved cannot be determined. For the alkoxy substituents, molar refractivity (MR) and Verloop's L parameter, both measures of substituent size, are colinear with hydrophobic substituent parameter, π. All parameters give a good correlation with the pIC_{50} data for the alkloxy substituents (Table 2, Equations 1, 2 and 3).

Branching of the alkoxy substituent in the β-position (that is, the position adjacent to the atom directly attached to the benzimidazole) reduced activity. Thus, the *iso*-propoxy compound (IV, 12, $IC_{50} = 7.4$ μM) had one-third the activity of the *n*-propoxy analogue indicating that the shape of the substituent may also be important for activity.

To examine further the possible effect of the shape of the substituent on activity, two alkyl substituents branched in the α-position (adjacent to the benzimidazole ring) were examined. The *iso*-propyl compound (IV, 22) was shown to be about seven times less potent than its *n*-propyl analogue (IV, 21). Further branching in the α-position resulted in still further loss of activity, compound IV, 24 being about three times less potent than IV, 22. Hence, the extent of branching in either the α- or β-positions is a determinant for potency. This may be due to an unfavourable steric interaction with the receptor since, in the more highly branched substituents, differences in the hydrophobic (π) and electronic characteristics (σp and σm) are minimal (compare IV, 12 with IV, 11, IV, 22 with IV, 21 and IV, 24 with IV, 23). As no loss in potency was observed for the γ-branched substituents, it must be considered that the steric interaction is of importance only in the α- and β-positions, a distance of approximately

Table 2. Regression analysis of the inhibition of the rate of polymerisation of tubulin by benzimidazole carbamates

	r*	SD†	Equation
(1) Eight compounds (Table 1, Compounds IV, 9–11, 13, 15–18)			
$pIC_{50} = 1.07\,\pi - 0.23\,\pi^2 + 4.51$	0.93	0.21	1
$pIC_{50} = 1.66\,MR - 0.28\,MR^2 + 3.32$	0.94	0.20	2
$pIC_{50} = 9.92\,L - 5.70\,L^2 + 1.41$	0.93	0.20	3
(2) Twenty-four compounds (Table 1, Compounds IV, 1, 2, 5–7, 9–21, 23, 25, 26, 28, 30, 32)			
$pIC_{50} = 0.39\,\pi + 4.66$	0.76	0.32	4
$pIC_{50} = 1.15\,\pi - 0.24\,\pi^2 + 4.28$	0.90	0.21	5
$pIC_{50} = 1.06\,\pi - 0.24\,\pi^2 + 0.29\,I_{alkyl} + 4.28$	0.94	0.18	6
$pIC_{50} = 0.36\,MR + 4.59$	0.73	0.34	7
$pIC_{50} = 0.99\,MR - 0.17\,MR^2 + 4.13$	0.85	0.27	8
$pIC_{50} = 0.84\,MR - 0.15\,MR^2 + 0.31\,I_{alkyl} + 4.17$	0.90	0.23	9

* Multiple correlation coefficient.
† Standard deviation from the regression.

4 Å from the benzimidazole ring in the 5 (or 6) position.

Steric interactions in the α- and β-positions appear to depend not only on the extent of branching, but also on the molecular geometry of the moieties in the α- and β-positions. Thus, the β-branched, sp^2 substituents such as phenoxy (IV, 25) and phenylthio (IV, 26) are significantly (2-fold) more potent than the sp^3 hybridised *iso*-propoxy substituents, while the α-branched benzoyl moiety (sp^2 hybridised) is also a potent inhibitor.

That the inhibitory activity is not dependent simply on steric parameters at the α- and β-positions is demonstrated by a comparison of fenbendazole (IV, 26) and its sulphoxide analogue (IV, 27). In both these compounds the sulphur atoms are sp^3 hybridised, but oxidation of the sulphur has increased the polarity of the α-position substantially. Since π is a logarithmically additive substituent parameter, the difference in the π values of $\pi_{SC_6H_5} = 2.35$ and $\pi_{SOC_6H_5} = 0.07$ indicates that the polarity in the α-position has increased 100 times on branching. This polarity shift resulted in the total loss of activity of (IV, 27). Similarly, reduction of the keto group of mebendazole (IV, 28) to the phenylcarbinol (IV, 29), altering the molecular geometry from sp^2 to sp^3, resulted in a 5-fold more polar substituent ($\pi_{COC_6H_5} = 1.05$, $\pi_{CHOHC_6H_5} = 0.54$). This change resulted in an 11.5-fold loss in potency.

In considering the importance of modification of the 5(6)-substituent in the α- or β-positions, it can be seen that not only are the extent of branching and the molecular geometry important for activity, but so also is the polarity of this region. In fact, it would appear that the presence of a polar moiety in this region of the molecule is significantly more important than either the geometry or branching of the substituent.

Quantitative structure–activity relationships were derived from this study to enable some conclusions to be drawn about a model for interaction of the 5(6)-substituent with tubulin. Of the thirty-two compounds examined in Table 1, compounds (IV, 3, 4, 8 and 27) were excluded as they failed to inhibit polymerisation. Nocodazole (IV, 31) and α-branched compounds (IV, 22, 24 and 29) were excluded as insufficient information was available to quantitatively describe the substituent.

Initial correlation of the remaining twenty-four compounds to the hydrophobic substituent parameter, π (Table 2, Equation 4) gave a correlation coefficient of 0.76, accounting for 57% of the variance of the pIC_{50} values. The inclusion of π^2 term significantly ($P < 0.05$) improved the regression ($r = 0.90$), demonstrating the parabolic dependence of hydrophobicity on the inhibition of polymerisation. Examination of molar refractivity as a "corrected" molar volume [25] gave comparable correlations to π for both the linear (Equation 7) and parabolic (Equation 8) models. Although the correlation coefficient for the parabolic hydrophobicity is superior ($r = 0.90$) compared with the molar volume model ($r = 0.85$), the high correlation of the latter model indicates that the site of interaction in the 5(6)-position may not be completely hydrophobic in nature. The failure of the small polar substituents (IV, 3, 4, and 8) and large substituents with polar groups in the α-position (IV, 27 and 29) to inhibit polymerization can be explained based on the parabolic hydrophobicity model. However, the possibility that the presence of a polar substituent in the α- and β-positions represents a specific interaction analogous to the effects of branching cannot be discounted.*

To test whether the hydrophobicity model or the

* Recent studies with 3′- and 4′-hydroxyphenoxy-, and 3′- and 4′-aminophenoxy-substituents which are all potent inhibitors suggested that an increase in polarity in the α-position is a specifically undesirable interaction (E. Lacey and T. R. Watson, unpublished results).

molar volume model best represents the nature of the interaction of the substituent with tubulin, it would be necessary to examine a number of large polar substituents with no branching or polar substituents in the α-position. This study is currently under investigation.

The inclusion of electronic (σ_m or σ_p) or steric (Es and B_1) substituent parameters did not improve significantly either the hydrophobic or molar volume models. However, the inclusion of an indicator variable (I_{alkyl}) for the presence of an alkyl group attached to the benzimidazole ring by either an oxygen or methylene bridge of minimum $—CH_2CH_2—R$ or $—OCH_2—R$ gave a significant improvement in both models (Equations 6 and 9). The introduction of I_{alkyl} into both parabolic models accounted for 88% and 80% of the variance of the pIC_{50} data for the hydrophobic and molar volume models respectively. At the level of the molecular interaction of these substituents with tubulin, it is probable that the greater conformation flexibility of these substituents could enable a better "fit" to the site compared with that of the new conformationally restrained phenoxy (IV, 25), phenylthio (IV, 26) and benzoyl (IV, 28).

Although the models derived in this study clearly demonstrate the role of the hydrophobicity and/or size of the 5(6)-substituent on activity, the lack of availability of parameters to describe the steric or polar characteristics in the α-position requires that caution be exercised in predicting activity of such substituents using these models. While this interaction still requires further clarification to fully describe a quantitative model, the structure–activity relationships derived in this study represent not only an important contribution to the understanding of the benzimidazole–tubulin interaction but also form a basis for the investigation of the pharmacological specificity of these compounds as either antitumour, antifungal or anthelmintic agents. Studies to examine the role of tubulin and the structural specificity of benzimidazoles in exerting these actions are under investigation in this laboratory.

REFERENCES

1. P. Dustin (Ed.), *Microtubules*, Springer, Berlin (1978).
2. K. Roberts and J. S. Hyams (Eds.), *Microtubules*, Academic Press, New York (1979).
3. T. J. Fitzgerald, *Biochem. Pharmac.* **25**, 1383 (1976).
4. F. R. Quinn and J. A. Beisler, *J. med. Chem.* **24**, 251 (1981).
5. F. Zavala, D. Guenard and P. Potier, *Experientia* **34**, 1497 (1978).
6. S. M. Kupchan, A. T. Sneden, A. R. Branfman, G. A. Howie, L. I. Rebhun, W. E. McIvor, R. W. Wang and T. C. Schnautman, *J. med. Chem.* **21**, 31 (1978).
7. G. P. Clemons and H. D. Sisler, *Phytopathology* **59**, 705 (1969).
8. C. J. Di Cuollo, J. A. Miller, W. L. Mendelson and J. F. Pagaro, *J. agric. Fd. Chem.* **22**, 948 (1974).
9. V. J. Theodorides, J. Chang, C. J. Di Cuollo, G. M. Grass, R. G. Parish and G. C. Scott, *Br. vet. J.* **129**, xcvii (1973).
10. J. A. Brugmans, D. Thienpont, I. Van Wijngaarden, O. F. Vanparijs, V. L. Schuermans and H. L. Lauwer, *J. Am. med. Ass.* **217**, 313 (1971).
11. C. Baeder, H. Bahr, O. Christ, D. Duwel, H. M. Kellner, R. Kirsch, H. Loewe, E. Schultes, E. Schutz and H. Westen, *Experientia* **30**, 753 (1976).
12. V. J. Theodorides, R. J. Gyurik, W. D. Kingsbury and L. C. Parish, *Experientia* **32**, 702 (1976).
13. E. A. Averkin, C. C. Beard, C. A. Dvorak, J. A. Edwards, J. H. Fried, J. G. Kilian, R. A. Schitz, T. P. Kistner, J. H. Drudge, E. T. Lyons, M. L. Sharp and R. M. Corwin, *J. med. Chem.* **18**, 1164 (1975).
14. P. A. Friedman and E. G. Platzer, *Biochim. biophys. Acta* **544**, 605 (1978).
15. C. M. Ireland, K. Gull, W. E. Gutteridge and C. I. Podson, *Biochem. Pharmac.* **28**, 2680 (1979).
16. J. P. Laclette, G. Guerra and C. Zetina, *Biochem. biophys. Res. Commun.* **92**, 417 (1980).
17. N. P. Buv-Hoi, N. D. Xuong and N. H. Nam, *J. chem. Soc.* 1573 (1955).
18. P. E. Fanta and P. S. Tarbell, *Organic Synthesis Collective Volume III* (Ed. E. C. Horning), p. 661. John Wiley, New York (1955).
19. B. M. Lynch, C. M. Chen and Y-Y. Wigfield, *Can. J. Chem.* **46**, 1141 (1968).
20. S. H. Dandegaanker and D. Shastri, *Ml. Chem.* **96**, 614 (1965).
21. R. D. Haugwitz and B. V. Maurer, U. S. Patent No. 4,146,908 (1977); *Chem. Abstract.* **88**, 6883d (1978).
22. R. J. Allen, H. T. Goodman and T. R. Watson, *J. Chromat.* **183**, 614 (1980).
23. M. L. Shelanski, F. Gaskin and C. R. Cantor, *Proc. natn. Acad. Sci. U.S.A.* **70**, 765 (1973).
24. J. Hoebeke and G. Van Nijen, *Life. Sci.* **17**, 591 (1975).
25. C. Hansch and A. Leo, *Substituent Constants for Correlation Analysis in Chemistry and Biology*. Wiley Interscience, New York (1979).
26. A. Verloop, W. Hoogenstraaten and J. Tipker, in *Drug Design* (Ed. E. J. Ariens), Vol. III, pp. 165–207. Academic Press, New York (1975).
27. P. Veng Pedersen, *J. Pharmacokinetics Biopharm.* **5**, 513 (1977).