Journal of Natural Products
Vol. 58, No. 6, pp. 823-829, June 1995

FLAVONOID INHIBITORS OF TRYPSIN AND LEUCINE AMINOPEPTIDASE: A PROPOSED MATHEMATICAL MODEL FOR IC_{50} ESTIMATION

JOSEFINA PARELLADA

Department of Nutrition and Food Sciences,

and MARÍA GUINEA*

Department of Pharmacy and Pharmaceutical Technology, University of Alcalá de Henares, E-28871 Alcalá de Henares, Madrid, Spain

ABSTRACT.—The inhibitory behavior of flavonoids against trypsin and leucine aminopeptidase followed sigmoidal curves similar to those of any dose-biological response relationship. Statistical analysis using several mathematical equations showed that the relationship may be expressed by a logistic equation, which yielded a high correlation between the experimental data and the predicted results, together with an objective criterion for estimating the IC_{50} value. Flavones and flavonols exhibited a strong inhibitory effect on trypsin; the presence of hydroxyl groups at positions C-5 and C-7 in ring A is necessary for inhibition of the enzyme, while the simultaneous presence of free hydroxyl groups at positions C-3′ and C-4′ enhances the inhibitory activity. Inhibition of leucine aminopeptidase by flavonoids does not require 5,7-hydroxylation, but dihydroxylation at C-3′ and C-4′ and a double bond at positions C-2, C-3 are essential for this activity.

Flavonoids are commonly ingested in fruits and vegetables in the human diet. They have no nutritive value but are capable of exerting a broad range of antiallergic, antihypertensive, anti-inflammatory, antimicrobial, antimutagenic, antineoplastic, antiulcerogenic, antiviral, hepatoprotective, and hypolipidemic effects (1–7); anti-HIV activity has also been reported recently (8). Certain flavonoids are known to interfere with several enzymes implicated in pathological processes, such as adenosine deaminase (9), AMP_c phosphodiesterase (10), angiotensin-converting enzyme (2), cyclooxygenase and lipoxygenase (11), protein tyrosine kinase (12), and HIV-1 proteinase (13).

Previous work has also shown flavonoids to be capable of inhibitory activity against a variety of proteases. The present study compares the inhibitory activity of eleven flavonoids on two proteases involved in a variety of pathological processes: trypsin, a serine proteinase strongly implicated in acute pancreatitis (14), inflammatory processes (15,16), and in various stages of tumorous invasion (17); and leucine aminopeptidase, a membrane metalloprotease implicated in muscular dystrophy (18) and involved in various cancers (19–22).

In order to compare the inhibitory properties of the different flavonoids considered, and to establish the possible relationships between structure and activity, the IC_{50} values for several enzyme inhibitory flavonoids were determined. Typically, quantification of this parameter has not been defined by mathematical modeling but has been left to the discretion of each individual researcher. Inhibitory concentration 50% (IC_{50}) has therefore been estimated by others using direct interpolation from dose-response curves (23–25), regression analysis (26), or probit transformation (9,27); many previous papers do not provide any description of the estimation method used. In the authors' view, a mathematical expression that will fit experimental results is needed to standardize IC_{50} values and therefore furnish comparable results.

Like all biological dose-response relationships, the concentration-inhibition relationship follows a sigmoidal curve (28), and hence the statistical analysis of the experimental results was carried out using nonlinear regression, applying equations for three mathematical criteria that fit sigmoidal curves, namely, the logistic, Gompertz,

and Weibull equations (29). The fitness of the different models was established by comparing the coefficient of determination (r^2) values for the nonlinear equations, as well as linear regression and probit transformation.

RESULTS AND DISCUSSION

All flavonoids tested inhibited trypsin, whereas only luteolin, 3′,4′-dihydroxyflavone, and quercetin inhibited leucine aminopeptidase. Progress curves for the enzymatic reactions in the presence of these inhibitors followed zero-order kinetic equations, and the reaction rates remained constant. Statistical analysis of the inhibition results by the different models yielded the coefficient of determination values (r^2) presented in Table 1.

Quercetin and myricetin strongly inhibited trypsin but were fit poorly by the linear model, which had r^2 values of only 22% for quercetin and 77% for myricetin. Linearization of the relation by means of probit transformation considerably improved the fit of the equation, yielding r^2 values of 45% for quercetin and 85% for myricetin, whereas application of the nonlinear equations raised the coefficient of determination values to around 70% for quercetin and 96% for myricetin. When the inhibitory response failed to reach the plateau on the sigmoidal curve, as in the case of inhibition of trypsin by apigenin, 3′,4′-dihydroxyflavone, taxifolin, kaempferol, morin, and silychristin (Figure 1), there were no appreciable differences in the fit of the linear and nonlinear models to the data, and linearization of the relationship by probit transformation lowered the r^2 values by between 15 and 35%. Thus, we can conclude that the nonlinear equations fit our experimental data much better than did linear regression and probit transformation.

The statistics of the three nonlinear models were very similar (data available from the authors upon request). For this reason, the choice of the best model had to rely on other considerations. We have considered the parameter K (the value of the asymptote where it crosses the y-axis) as the reference point to assess how well the models approximated the actual situation; therefore its maximum real value will be equal to or

TABLE 1. Coefficient of Determination (r^2) Values for the Different Statistical Procedures Applied to the Inhibition of Trypsin and Leucine Aminopeptidase by Flavonoids.

Flavonoid/Enzyme	r^2 (%)					
	Data number	Linear	Probit	Logistic	Gompertz	Weibull
Apigenin[a]	60	84.5	54.7	88.5	88.1	86.0
Luteolin[a]	93	65.6	57.2	75.4	77.0	79.9
3′,4′-Dihydroxyflavone[a]	84	76.0	65.0	73.2	75.6	79.8
Kaempferol[a]	68	91.2	73.9	90.5	91.1	89.9
Quercetin[a]	168	21.6	45.2	69.7	70.1	69.6
Morin[a]	75	83.9	56.9	84.2	84.3	80.5
Myricetin[a]	94	76.7	85.5	95.9	95.9	96.8
Eriodictyol[a]	53	61.8	67.7	91.8	91.9	90.4
Taxifolin[a]	53	56.5	69.0	59.2	56.7	59.2
Silychristin[a]	56	93.0	90.1	94.8	95.0	94.2
Luteolin[b]	77	67.5	82.9	72.9	75.8	78.0
3′,4′-Dihydroxyflavone[b]	90	63.9	42.2	84.6	84.6	78.9
Quercetin[b]	114	70.7	87.2	80.8	82.5	89.9

[a]Trypsin.
[b]Leucine aminopeptidase.

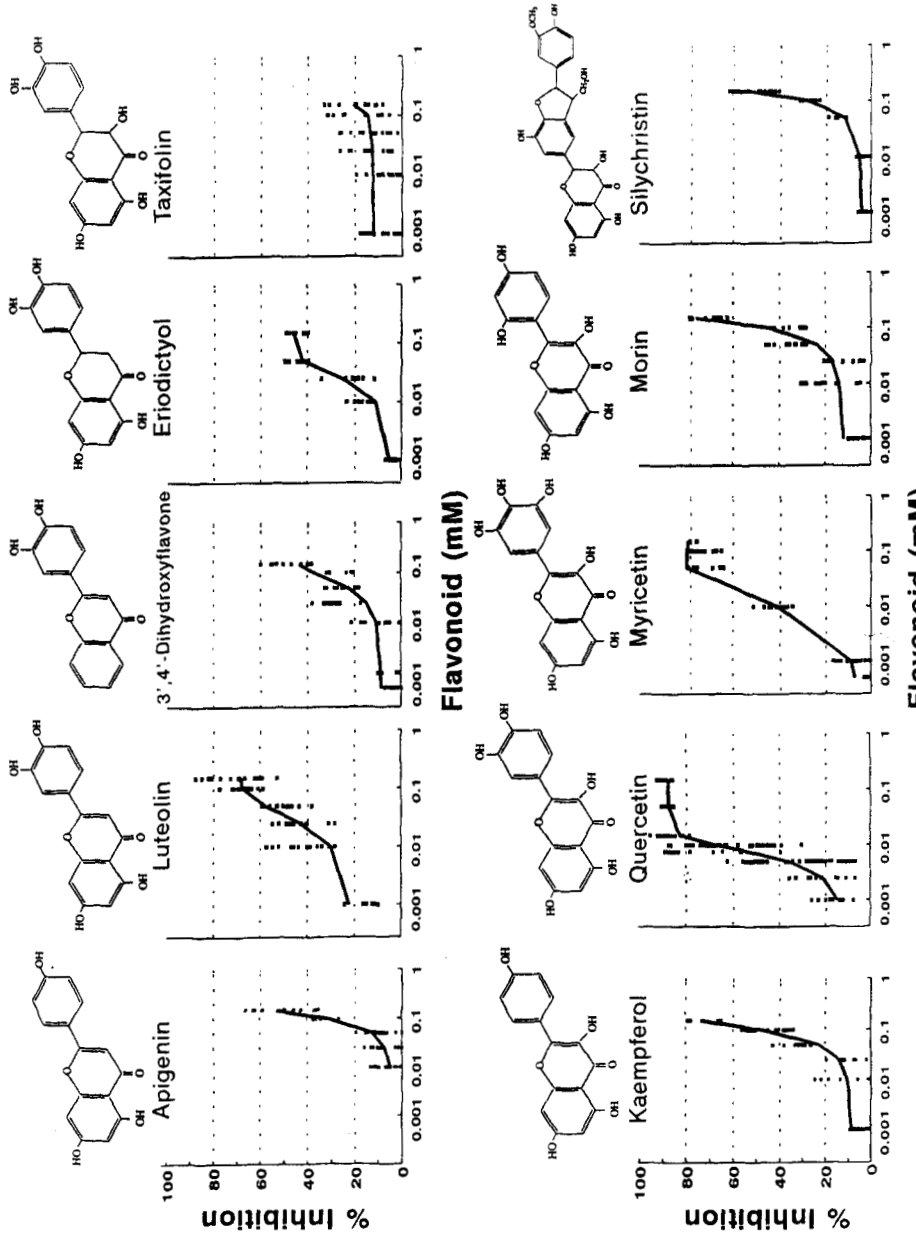

FIGURE 1. Trypsin inhibition by flavonoids: Dose-response curves of active compounds fitted by the logistic equation.

TABLE 2. Estimated Values for the Parameter *K* Obtained for Flavonoid Enzyme Inhibitors Using Three Equations.

Flavonoid/Enzyme	*K*		
	Logistic	Gompertz	Weibull
Apigenin[a]	71.8	162.1	303.1
Luteolin[a]	68.6	68.7	199.8
3′,4′-Dihydroxyflavone[a]	45.7	44.4	237.8
Kaempferol[a]	99.1	141.5	485.8
Quercetin[a]	87.7	89.5	90.1
Morin[a]	240.1	137.4	207.6
Myricetin[a]	95.4	95.5	99.3
Eriodictyol[a]	45.8	46.2	46.3
Taxifolin[a]	36.5	48.8	25.3
Silychristin[a]	97.8	222.8	469.5
Luteolin[b]	69.9	67.4	80.1
3′,4′-Dihydroxyflavone[b]	56.6	56.6	94.9
Quercetin[b]	66.1	70.1	185.1

[a]Trypsin.
[b]Leucine aminopeptidase.

less than 100%. Based on the values of *K* (Table 2), the logistic model provided the best reflection of the experimental data, and the concentration-inhibition curves fit by that model were sigmoidal (Figures 1 and 2), with asymptote values in the vicinity of 100% for the most potent inhibitors. For the weakest inhibitors asymptote values corresponded to inhibition rates of less than 50%, i.e., in the inhibition of trypsin by eriodictyol, for which it was not possible to define the IC_{50} value. Over the range of concentrations tested, the activity of other inhibitors was limited to the initial latency phase (e.g., inhibition of trypsin by taxifolin). Here again the IC_{50} value could not be determined. Inhibition of trypsin by apigenin, morin, kaempferol, and silychristin attained but did not go beyond the exponential response phase. We consider the ability to predict the IC_{50} value for these last-mentioned inhibitors to be of high interest, since under normal experimental conditions difficulties relating to solubility and other aspects generally prevent higher concentrations from being tested (9).

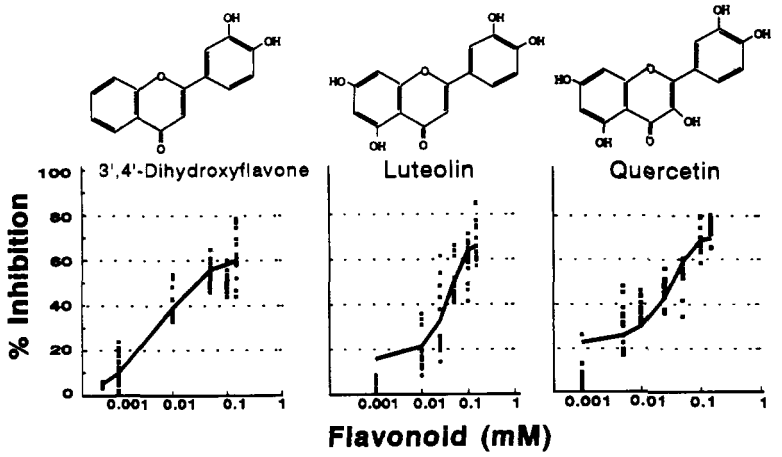

FIGURE 2. Leucine aminopeptidase inhibition by flavonoids: Dose-response curves of active compounds fitted by the logistic equation.

To this end, the experimental curves for those inhibitors that reached levels of complete inhibition, i.e., inhibition of trypsin and leucine aminopeptidase by luteolin and quercetin, were divided into two intervals. The first interval included the latency phase and the linear phase, while the second interval included the linear phase and the asymptotic phase. The inhibition results over each interval were treated mathematically using the logistic equation, and the IC_{50} value was calculated for each interval by means of the following expression, solving the logistic equation for IC_{50}.

$$IC_{50} = \frac{\ln \frac{\frac{K}{50} - 1}{c}}{-a}$$

The values of IC_{50} so calculated were of the same order of magnitude, irrespective of whether the entire curve or only one interval was considered (Table 3). Variability was between 6 and 18%, hence the logistic model fit the experimental data as well as predicting the IC_{50} values for the enzyme inhibitors when only a portion of the data for plotting the curves was available, thus yielding fully comparable values.

TABLE 3. Estimates of IC_{50} Values for Different Intervals on the Sigmoidal Curve Using the Logistic Equation for Flavonoid Enzyme Inhibitors.

Flavonoid/Enzyme	IC_{50} μM ($\pm\sigma_n$)		
	Interval I	Interval II	Combined
Luteolin[a]	33.5 (±7) r^2=75.9	41.3 (±5) r^2=68.7	35.4 (±6) r^2=75.4
Luteolin[b]	52.5 (±17) r^2=61.6	58.6 (±8) r^2=55.6	49.6 (±13) r^2=74.8
Quercetin[a]	5.9 (±3) r^2=69.7	6.2 (±1) r^2=68.9	7.1 (±2) r^2=69.7
Quercetin[b]	31.2 (±7) r^2=82.1	36.7 (±3) r^2=77.9	34.3 (±4) r^2=80.8

[a]Trypsin.
[b]Leucine aminopeptidase.

The IC_{50} values for the inhibitory power of the different flavonoids against trypsin (Table 4) suggest that to inhibit this serine proteinase the flavonoids must meet certain structural requirements. The presence of hydroxyl groups at C-5 and C-7 in ring A appears to be essential for inhibitory activity. These hydroxyl groups may be involved in the formation of hydrogen bridges between the flavonoid and the amino acid residues located near or at the active site on the trypsin molecule. Such interactions have been proposed for flavonoids that inhibit HIV-proteinase (13). The double bond at position C-2,C-3 in ring C and hydroxylation at both the C-3′ and C-4′ positions help augment inhibitory activity.

In the inhibition of leucine aminopeptidase by flavonoids, only those compounds with a double bond at C-2,C-3 showed activity (Figure 2 and Table 5). This double bond confers on the flavonoids certain conformational attributes that enable them to interact with cell membranes (30) and such a conformation seems to be required to inhibit this membrane metallopeptidase. Flavones and flavonols dihydroxylated at the C-3′ and C-4′ positions were the most active substances, hence their inhibitory activity may be related to bonding of their ortho-dihydroxyl groups to the zinc atom or atoms at the active site on the enzyme (31), as in the inhibition of other metalloenzymes (32).

TABLE 4. IC_{50} Values for Trypsin-Inhibiting Flavonoids.

Flavonoid	IC_{50} μM ($\pm\sigma_n$)
Apigenin	141.5 (±17)
Luteolin	35.3 (±6)
Kaempferol	105.9 (±22)
Quercetin	7.1 (±2)
Morin	110.8 (±33)
Myricetin	10.2 (±2)
Silychristin	144.4 (±18)

TABLE 5. IC_{50} Values for the Leucine Aminopeptidase-Inhibiting Flavonoids.

Flavonoid	IC_{50} μM ($\pm\sigma_n$)
3′,4′-Dihydroxyflavone	35.1 (±4)
Luteolin	49.6 (±13)
Quercetin	34.3 (±4)

EXPERIMENTAL

CHEMICALS.—Apigenin, 3′,4′-dihydroxyflavone, eriodictyol, kaempferol, luteolin, and myricetin were obtained commercially from Extrasynthèse, Lyon, France; taxifolin and quercetin from Sigma Chemical Co., Madrid, Spain, and morin from Merck, Madrid, Spain; silybin and silychristin were kindly supplied by Inverni della Beffa, Milan, Italy. These flavonoids were dissolved in MeOH or EtOH and then diluted with H_2O before being used in each assay. The range of concentrations was 1–150 μM and at least five different concentrations were tested for each compound. The maximum alcohol concentration in the enzymatic assays was 6%.

Trypsin (E.C.3.4.21.4) type III from bovine pancreas and leucine aminopeptidase (EC 3.4.11.1) from pork kidney cytosol were obtained from Sigma Chemical Co. (Madrid, Spain). The substrates *N*-α-benzoyl-DL-arginine-*p*-nitroanilide and L-leucine-*p*-nitroanilide were also purchased from Sigma Chemical Co. (Madrid, Spain).

TRYPSIN ACTIVITY.—One unit was dissolved in 100 ml of 1 mM HCl. The substrate, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (20 mM), was prepared in 0.2 M TEA-HCl buffer, pH 7.8, with 65% DMSO (33). Aliquots of enzyme (0.26 ml) and buffer (4.6 ml), with and without test compound, were preincubated at 37° for 30 min. The enzymatic reaction was started by adding substrate (0.26 ml). Samples of all reaction mixtures were collected, diluted, and measured spectrophotometrically at 405 nm at 5 time intervals during the reaction period, to determine the amount of *p*-nitroaniline released. Blank samples were prepared with 0.26 ml of 1 mM HCl instead of trypsin. Four replicates of all samples were evaluated in each assay.

LEUCINE AMINOPEPTIDASE ACTIVITY.—One unit was dissolved in 100 ml of 0.2 M TEA-HCl buffer, pH 7.8, with 5 mM of $MgCl_2$. The substrate, L-leucine-*p*-nitroanilide (20 mM), was prepared in buffer with 4.2% DMFA (34). Aliquots of enzyme (0.446 ml) and buffer (2 ml), with and without test compounds, were preincubated at 37° for 30 min. The enzymatic reaction was started by adding substrate (0.154 ml), and sample collection and measurement followed the procedure described above for trypsin.

INHIBITORY ACTIVITY.—Inhibitory activity was estimated using the equation $IA=100-100(V_t/V_c)$ (35), where V_t is the enzymatic reaction velocity in the presence of a test compound and V_c is the velocity in the control assay.

STATISTICAL TREATMENT.—Concentration and percentage inhibition were analyzed statistically by linear regression, probit transformation, and nonlinear regression analysis using the Gompertz [1], Weibull [2], and logistic [3] equations (29), as follows:

$$[1] \quad y=K\exp[-a\exp(-bx)]$$

$$[2] \quad y=K[1-\exp(-bx^c)]$$

$$[3] \quad y=\frac{K}{1+c\exp(-ax)}$$

All calculations were performed using the Statgraphics (Statistical Graphics Corporation) computer program.

ACKNOWLEDGMENTS

We are grateful to Dr. J. Guinea (Pharma Mar S.A.) for his helpful discussions concerning statistics. This study was supported by the University of Alcalá de Henares.

LITERATURE CITED

1. H. Wagner, *Planta Med.*, **55**, 235 (1989).
2. H. Wagner, G. Elbl, H. Lotter, and M. Guinea, *Pharm. Pharmacol. Lett.*, **1**, 15 (1991).
3. M. Gabor, in: "Handbook of Experimental Pharmacology-Anti-Inflammatory Drugs." Ed. by J.R. Vane and S.H. Ferreira, Springer-Verlag, New York, 1979, Vol. 50, Chapter 39, pp. 698–739.
4. N. Kaneda, J.M. Pezzuto, D.D. Soejarto, A.D. Kinghorn, and N.R. Farnsworth, *J. Nat. Prod.*, **54**, 196 (1991).
5. A.J. Vlietinck, D.A. Vanden Berghe, and A. Haemers, in: " Plant Flavonoids in Biology and Medicine II: Biochemical, Cellular, and Medicinal Properties." Ed. by V. Cody, E. Middleton, Jr., J.B. Harborne, and A. Beretz, Alan R. Liss, New York, 1988, Vol. 280, pp. 283–299.
6. H. Hikino and Y. Kiso, in: "Economic and Medicinal Plant Research," Ed. by H. Wagner, H. Hikino, and N.R. Farnsworth, Academic Press, London, 1988, Vol. 2, pp. 39–72.
7. M.A.F. Jahromi, A.B. Ray, and J.P.N. Chansouria, *J. Nat. Prod.*, **56**, 989 (1993).
8. C. Hu, K. Chen, Q. Shi, R.E. Kilkuskie, Y. Cheng, and K.-H. Lee, *J. Nat. Prod.*, **57**, 42 (1994).
9. H.P. Koch, W. Jäger, U. Groh, and G. Plank, *Meth. Find. Exp. Clin. Pharmacol.*, **14**, 413 (1992).
10. T. Nikaido, T. Ohmoro, U. Sankawa, T. Hamanaka, and K. Totsuka, *Planta Med.*, **46**, 162 (1982).
11. K. Sekiya and H. Okuda, *Biochem. Biophys. Res. Commun.*, **105**, 1090 (1982).
12. M. Abou-Shoer, G.-E. Ma, X.-H. Li, N.M. Koonchanok, R.L. Geahlen, and C.-J. Chang, *J. Nat. Prod.*, **56**, 967 (1993).
13. R.I. Brinkworth, M.J. Stoermer, and P. Fairlie, *Biochem. Biophys. Res. Commun.*, **188**, 631 (1992).
14. C. Figarella, M. Amouric, and O.G. Crotte, in: "Acute Pancreatitis: Research and Clinical Management." Ed. by H.G. Berger and M. Büchler, Springer-Verlag, Berlin, 1987, pp. 53–60.
15. M.R. Ariño, M. Catalán, J.R. Azanza, and J. Honorato, *Dolor Inflamación*, **5**, 361 (1988).
16. S.M. Wahl, in: "Methods in Enzymology. Immunochemical Techniques." Ed. by G.D. Sabato, Academic Press, San Diego, 1988, Vol. 163, pp. 605–622.
17. H. Tschesche and H.W. Macartney, in: "Proteinase Inhibitors: Medical and Biological Aspects," Ed. by N. Katunuma, H. Umezawa, and H. Holzer, Springer-Verlag, Berlin, 1983, pp. 211–228.
18. R.K. Day and C. Ashmore, *Muscle Nerve*, **7**, 482 (1984).
19. S.K. Gupta, M. Aziz, and A.A. Khan, *Ind. J. Pathol. Microbiol.*, **32**, 301 (1989).
20. M. Aziz, S.K. Gupta, and A.A. Khan, *J. Ind. Med. Assoc.*, **88**, 160 (1990).
21. R.R. Rackley, B. Yang, T.G. Pretlow, F.W. Abdul-Karim, T.J. Lewis, N. McNamara, C.M. Delmoro, E.L. Bradley, Jr., E. Kursh, and M.I. Resnick, *Cancer*, **68**, 587 (1991).
22. M.M. Khanolkar, A.V. Sirsat, V.H. Deshmane, and M.R. Kamat, *Ind. J. Med. Res.*, **96**, 372 (1992).
23. M. Bols, L. Binderup, J. Hansen, and P. Rasmussen, *J. Med. Chem.*, **35**, 2768 (1992).
24. K. Yasukawa, M. Takido, M. Takeuchi, and S. Nakagawa, *Chem. Pharm. Bull.*, **37**, 1071 (1989).
25. E. Olsson, A. Holth, E. Kumblin, L. Bohlin, and I. Wahlberg, *Planta Med.*, **59**, 293 (1993).
26. T. Pengsuparp, L. Cai, H.S. Fong, A.D. Kinghorn, J.M. Pezzuto, M.C. Wani, and M.E. Wall, *J. Nat. Prod.*, **57**, 415 (1994).
27. H.P. Koch and E. Löffler, *Meth. Find. Exp. Clin. Pharmacol.*, **7**, 13 (1985).
28. E. Perucca and F. Pisani, in: "Dose-Response Relationships in Clinical Pharmacology." Ed. by L. Lasagna, S. Erill, and C.A. Naranjo, *Excerpta Medica*, Amsterdam, 1989, pp. 201–214.
29. J.D. Lebreton and C. Millier (Eds.), "Modéles Dynamiques Déterministes en Biologie." Masson, Paris, 1982.
30. R.M. Price and E. Middleton, Jr., in: "Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships." Ed. by V. Cody, E. Middleton, Jr., and J.B. Harborne, Alan R. Liss, New York, 1986, Vol. 213, pp. 507–510.
31. H. Kim and W.N. Lipscomb, *Biochemistry*, **32**, 8465 (1993).
32. H. Zollner, "Handbook of Enzyme Inhibitors," VCM Verlagsgesellschaft mbH, Weinheim, Germany, 1989.
33. R. Geiger and H. Fritz, in: "Methods of Enzymatic Analysis." Ed. by H.V. Bergmeyer, J. Bergmeyer, and M. Graßl, Verlag-Chemie, Basel, 1984, Vol. V, pp. 119–129.
34. R.J.P. Cannell, S.J. Kellam, A.M. Owsianka, and J.M. Walker, *Planta Med.*, **54**, 10 (1988).
35. M.C. Guinea, J. Parellada, M.A. Lacaille-Dubois, and H. Wagner, *Planta Med.*, **60**, 99 (1994).

Received 26 May 1994