Phytochemistry, Vol. 25, No. 3, pp. 589–593, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00
Pergamon Press Ltd.

SPECIFICITY OF ADENINE BINDING TO LIMA BEAN LECTIN*

DAVID D. ROBERTS†, PALANISAMY ARJUNAN, LEROY B. TOWNSEND and IRWIN J. GOLDSTEIN

Departments of Biological Chemistry and Medicinal Chemistry, The University of Michigan, Ann Arbor, MI 48109, U.S.A.

(*Received* 29 *March* 1985)

Key Word Index—*Phaseolus lunatus*; Leguminosae; lima bean; lectin binding; purines; adenine.

Abstract—The interactions between lima bean lectin (LBL) and adenine were examined using a series of synthetic purine analogs. Binding was sensitive to modification at most positions of the purine ring, suggesting a high degree of specificity for adenine binding. Methylation of the 6 NH_2-group to MeNH-, Me_2N- and Me_3N^+-analogs progressively decreased the binding affinity. Compounds lacking the 6 NH_2-group were not bound. Methylation of adenine at N^1, N^3 or N^7 also inhibited binding, indicating specific interactions with these ring nitrogens. In contrast to the previous report that N^9-substituted adenines, nucleosides and nucleotides were not bound [Roberts, D. D. and Goldstein, I. J. (1983) *J. Biol. Chem.* **258**, 13820], 9-methyl- and 9-benzyl-substituted adenines were bound to LBL with high affinity. Substitutions at C-2 and C-8 were tolerated and, in some cases, increased the affinity of binding to LBL. Heterotropic interactions between the adenine and 1,8-anilinonaphthalenesulphonate binding sites were also sensitive to modification of the purine ring. 2-Methylthioadenine and 4-aminopyrazolo[3,4-*d*]pyrimidine showed increased allosteric interaction with 1,8-anilinonaphthalenesulphonate binding, whereas several adenine analogs with a 9-*p*-nitrobenzyl substituent appeared to be negative effectors of 1,8-anilinonaphthalenesulphonate binding.

INTRODUCTION

The lectin from lima beans (*Phaseolus lunatus*) agglutinates human blood group A erythrocytes [1] and binds specifically to the nonreducing terminal trisaccharide sequence α-D-GalNAc(1 → 3) [α-LFuc(1 → 2)]-β-D-Gal which is present on the surface of these cells [2, 3]. Lima bean lectin (LBL) component III, a tetramer of 31 k M_r subunits [4, 5], has four carbohydrate binding sites [6]. The tetramer binds two moles of Ca^{2+} and two moles of Mn^{2+} [7], which are required for carbohydrate binding activity [4, 8]. LBL component III also contains four sites which bind the fluorescent hydrophobic ligand 1,8-anilinonaphthalenesulphonate (ANS) and one site which binds 2,6-toluidinylnaphthalenesulphonate, adenine and some analogs of adenine with cytokinin activity [9, 10].

Binding sites for nonpolar ligands are present in many lectins [11–14]. Almost all legume lectins bind ANS [14]. Adenine also binds to several legume lectins in addition to LBL [10]. The amino acid residues which form the hydrophobic binding sites on each subunit of concanavalin A, the lectin from *Canavalia ensiformis*, were identified by X-ray crystallographic analysis [15]. These residues are highly conserved in other lectins for which amino acid sequences are known [16–18], suggesting that the sites may be important for lectin function in legumes.

We have further examined the specificity of LBL [19] for adenine using a series of synthetic derivatives [20]. These studies demonstrate that the binding site of LBL is highly complementary to adenine and provide a basis for examining the interactions of other legume lectins with adenine and related metabolites.

RESULTS AND DISCUSSION

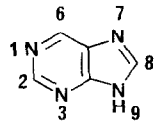
Binding of adenine analogs to LBL was measured by a fluorimetric assay based on allosteric enhancement of ANS binding [10]. Relative affinities derived from this assay were shown previously to correlate well with affinity constants measured using equilibrium dialysis for adenine, N^6-benzyladenine, zeatin and hypoxanthine [10]. Compounds which failed to bind as measured by the fluorescence assay also failed to bind using UV difference spectroscopy [10]. We therefore adopted the fluorescence assay to determine relative affinity constants for the synthetic adenine analogs (Table 1) which are summarized in Table 2. In agreement with previous results, most adenine analogs enhanced ANS binding to LBL. As shown in the representative titrations in Fig. 1, the degree of enhancement and binding affinity varied greatly. Furthermore, binding affinity did not correlate well with the magnitude of ANS fluorescence enhancement at saturating ligand. For example, 8-mercaptoadenine (**18**) ($K_{act} = 6.6 \times 10^{-6}$ M) bound with higher affinity than 4-aminopyrazolo[3,4-*d*]pyrimidine (**19**) ($K_{act} = 4.2 \times 10^{-5}$ M). However, 8-mercaptoadenine increased bound ANS fluorescence 31% at saturation but the weaker ligand, 4-aminopyrazolo[3,4-*d*]pyrimidine, increased ANS fluorescence *ca* 5-fold.

We have previously demonstrated that the enhancement of ANS fluorescence by adenine and 6-benzyladenine results from an allosteric increase in the affinity of LBL for ANS following binding of these ligands [10]. Adenine and 6-benzyladenine did not change the quantum yield of ANS bound to LBL. The allosteric interactions of 6-benzyladenine are greater than those of adenine, resulting in a greater increase in ANS fluorescence with 6-benzyladenine than with adenine. To determine whether the large effects of 4-aminopyrazolo[3,4-*d*]pyrimidine and 2-methylthioadenine (**11**) result entirely from allo-

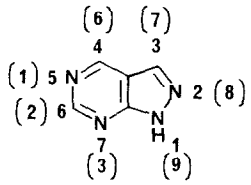
*Dedicated to Luis Leloir on his 80th birthday.

†Present address: Building 4, Room 327, National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20205, U.S.A.

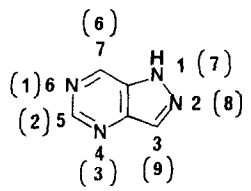
Table 1



Compound number	Substitution pattern 1	2	3	6	7	8	9
1				NHEt			Me
2		Cl		NHEt			
3				NHMe			
4				NMe_2			Me
5	Me			NHMe			
7		Cl		NHMe	Me		
8				NHMe			Ribose*
10				NH_2			Me
11		SMe		NH_2			
12		SH		NH_2			
13				SO_2NH_2			
14				NH_2		Cl	
15				NHOH			
16				$^+NMe_3$			
17		Me		NH_2			
18				NH_2		SH	
22		NH_2		NH_2			
23			Me	NHMe			
24				NH_2		I	
25				NH_2			$CH_2C_6H_5$
26				NH_2			$CH_2C_6H_4$-*p*-NO_2
27				NH_2		Br	$CH_2C_6H_5$
28				NH_2		N_2H_3	$CH_2C_6H_5$
29				NH_2		Br	$CH_2C_6H_4$-*p*-NO_2
30				NH_2		NH_2	$CH_2C_6H_5$
31				NH_2		N_2H_3	$CH_2C_6H_4$-*p*-NO_2



	5(1)†	6(2)	7(3)	4(6)	3(7)	2(8)	1(9)
9				NHEt			Me
19				NH_2			
20				NH_2	CN		



	6(1)†	5(2)	4(3)	7(6)	1(7)	2(8)	3(9)
21				Me			Me

* β-D-Ribofuranosyl.

† Numbers in parentheses refers to purine numbering.

Table 2. Effects of adenine derivatives on ANS binding to lima bean lectin

Ligand	$K_{activation}$ (M)	Effect on ANS fluorescence*
1	6×10^{-5}	+
2	3.6×10^{-5}	+
3	8×10^{-5}	+
4	2×10^{-4}	+
5	$> 10^{-3}$	+ (weak)
7	*ca* 1×10^{-3}	+ (weak)
8	$> 10^{-3}$	0
9	1×10^{-3}	+ (weak)
10	2.2×10^{-5}	+
11	1.5×10^{-5}	+ (strong)
12	3×10^{-4}	+
13	*ca* 1×10^{-3}	+ (weak)
14	1.2×10^{-5}	+
15	7×10^{-5}	+
16	$> 10^{-3}$	0
17	6.1×10^{-6}	+
18	6.6×10^{-6}	+
19	4.2×10^{-5}	+ (very strong)
20	7×10^{-5}	+
21	4.7×10^{-6}	+
22	8.9×10^{-6}	+
23	2.5×10^{-4}	+
24	1.3×10^{-5}	+
25	2.1×10^{-5}	+
26		−
27	*ca* 4×10^{-6}	+/−
28		− (weak)
29		−
30	1.0×10^{-5}	+/−
31		−
Purine	$> 10^{-3}$	+
Diphenylamine	1.2×10^{-4}	+
1-Naphthylamine	$> 10^{-3}$	+ (weak)
2-Naphthylamine	$> 10^{-3}$	− (weak)

*Ligands are classified by enhancement (+), inhibition (−), mixed enhancement and inhibition (+/−), or lack of effect (0) on fluorescence of ANS in the presence of LBL.

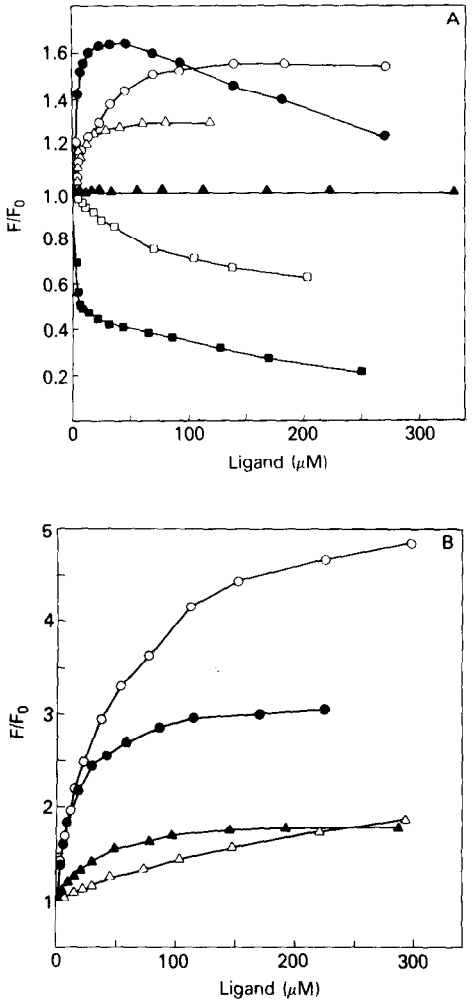


Fig. 1. Effect of adenine derivatives on ANS fluorescence in the presence of lima bean lectin. LBL containing 1×10^{-4} M ANS was titrated with the indicated ligands. After mixing and equilibration for at least 1 min at 25°, ANS fluorescence was read (λ_{ex} = 420 nm, λ_{em} = 480 nm). For each ligand, a parallel titration was performed by adding ligand to 1×10^{-4} M ANS in the absence of lectin. Corrected fluorescence (F) was calculated by subtraction of the blank fluorescence and multiplication by a dilution factor to correct for volume change due to the added ligand. Results were normalized by dividing by corrected fluorescence of ANS bound to LBL in the absence of ligand (Fo) and are presented as a function of concentration of ligand added. Panel A—9-benzyladenine **25** (○), 8-bromo-9-benzyladenine, **27** (●), 8-thioadenine, **18** (△), 6-methyladenosine, **8** (▲), 9-(4-nitrobenzyl)adenine, **26** (□), and 8-bromo-9(4-nitrobenzyl)adenine, **29** (■). Panel B—4-aminopyrazolo[3,4-*d*]-pyrimidine, **19** (○); 2-methylthioadenine, **11** (●), 3-methyl-6-methyladenine, **23** (△), and 6-benzyladenine (▲).

steric changes in the affinity of LBL for ANS, titrations of ANS were done in the presence and absence of these ligands (Fig. 2). The affinity of ANS binding is greatly increased by both ligands, but the quantum yield of ANS, which is proportional to the *x*-intercept on these plots, is unchanged. Thus the large variation in degree of ANS fluorescence enhancement with different ligands is due to a variation in the extent of heterotropic interaction with the ANS site.

Whereas all adenine derivatives examined previously, and most in the present study, are positive effectors of ANS binding, the 9-benzyl- and 9-(4-nitrobenzyl)adenine derivatives (**25**–**31**) inhibited ANS fluorescence. As we found for other ligands, the change in ANS fluorescence was seen only in the presence of LBL and is not due to direct interactions between the two ligands. A decrease in ANS fluorescence could result either from competitive inhibition of ANS binding to the four subunit sites or from negative heterotropic interactions between the adenine and ANS binding sites. These two possibilities cannot be distinguished on the basis of fluorescence titrations using ANS. Previously, we used a second fluorescent ligand, 2,6-toluidinylnaphthalenesulphonate (TNS), which binds with high affinity to the adenine site to identify the site to which 6-benzyladenine bound on LBL [10]. High affinity binding of TNS to the adenine site was selectively inhibited in the presence of 6-benzyladenine.

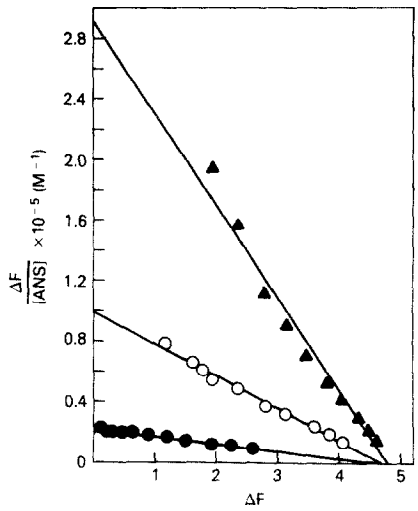


Fig. 2. Effect of 4-aminopyrazolo[3,4-*d*]pyrimidine and 2-methylthioadenine on ANS binding to lima bean lectin. Lima bean lectin (LBL) was titrated with 1,8-anilinonaphthalene-sulphonate (ANS) in the presence of 4-aminopyrazolo[3,4-*d*]pyrimidine, **19** (80 μM, ▲), 2-methylthioadenine, **11** (60 μM, ○), or in the absence of added ligands (●). The net increase in fluorescence (ΔF) was calculated by subtracting the fluorescence of free ANS measured in a parallel titration without LBL and correction for dilution due to added ligand. Results are presented in linearized form as described previously [10].

Similar experiments were conducted using three ligands which inhibited ANS fluorescence: 9-(4-nitrobenzyl)-adenine (**26**) (150 μM, a weak inhibitor), 8-bromo-9-benzyladenine (**27**) (24 μM, a mixed activator with weak inhibition), and 8 bromo-9-(4-nitrobenzyl)adenine (**29**) (10 μM, a potent inhibitor). All three ligands abolished high affinity TNS binding at the indicated concentrations (data not shown), suggesting that all three ligands bind to the adenine/high affinity TNS site. Thus the strong inhibition of ANS fluorescence observed at 1–10 μM for 8-bromo-9-(4-nitrobenzyl)adenine (**29**) appears to be an allosteric effect, as is the enhancement of ANS binding by 8-bromo-9-benzyladenine (**27**) at concentrations less than 25 μM.

High affinity binding of 8-bromo-9-(4-nitrobenzyl)-adenine to the adenine sites was confirmed by competitive equilibrium dialysis. This ligand inhibited binding of [^{14}C]-adenine to LBL with an apparent K_{dissoc} of 15 μM. Inhibition of ANS fluorescence by all three ligands at higher concentrations probably results from competition with ANS for the subunit sites. Weak competitive inhibition of ANS binding was noted previously with 6-benzyladenine [10].

The specificity of LBL for binding adenine was systematically examined by comparing the binding of adenine derivatives (Table 2). Introduction of a methyl group at N^1 of adenine abolished binding and at N^3 reduced the binding affinity. Purine, lacking the 6-amino group, was not bound, confirming the requirement for the 6-amino group proposed previously [10]. Progressive methylation of this group gave decreasing binding affinity in the order $NH_2 > NHMe > NMe_2 > N^+Me_3$. Other N^6-substituted adenine analogs including cytokinins bind to LBL with a preference for unsaturated side chains [10]. Substituents at position 2 were tolerated and in some cases enhanced binding. Both 2-methyladenine (**17**) and 2-aminoadenine (**22**) bound with higher affinity than adenine. Whereas 2-methylthioadenine (**11**) bound with the same affinity as adenine, 2-thioadenine bound only weakly. The low activity of 2-thioadenine (**12**) relative to 2-methylthioadenine (**11**) may be due to the stability of the 2-thione tautomer in the former. 2-Methylthio-derivatives are also much more active as cytokinins than are the corresponding 2-thio-derivatives [21].

Certain substituents at position 8 also enhanced binding to LBL. 8-Mercaptoadenine (**18**) bound 3-fold better than adenine. Both 8-chloro- (**14**) and 8-iodoadenine (**24**) were slightly more potent than adenine. As noted earlier, 8-substituents also modified the negative heterotropic interactions of 9-benzyladenines.

The role of N^7 in binding is uncertain at present. Comparing 2-chloro-N^6-methyl-N^7-methyladenine (**7**) (K_{act} *ca* 10^{-3} M) and 2-chloro-N^6-ethyladenine (**2**) ($K_{act} = 3.6 \times 10^{-5}$ M) suggests that methylation of N^7 inhibits binding. However, 4-amino-3-cyano-pyrazolo[3,4-*d*]pyrimidine (**20**) with a cyano group in the same position as this methyl group binds well.

The failure of nucleosides and nucleotides to bind to LBL would suggest that N^9 is necessary for binding. However, 9-methyladenine (**10**) bound as well as adenine, and some 9-benzyl- and 9-(4-nitro)benzyl-derivatives were good ligands. Thus, since these rather bulky groups on N^9 are tolerated, binding of nucleotides and nucleosides may be inhibited by unfavorable interactions of the highly polar sugar and phosphate groups with nonpolar protein amino acyl groups adjacent to N^9. Hydrophobic interactions between the 9-benzyl- groups and these residues may also account for their unusual effects on ANS binding.

The interactions of pyrazolopyrimidines with LBL are also noteworthy, with 4-amino-3-cyano-pyrazolo[3,4-*d*]pyrimidine (**19**) being bound with the greatest affinity of the adenine analogs tested ($K_{act} = 4.7 \times 10^{-6}$ M). 4-Aminopyrazolo[3,4-*d*]pyrimidine (**19**) bound with relatively high affinity but also produced the largest enhancement in ANS fluorescence. As shown in Fig. 2, this is due to increased heterotropic interactions, suggesting that contacts between the 7 or 8 positions and aminoacyl groups on LBL are important for the allosteric interactions between the two classes of sites. Binding of 4-ethylamino-1-methylpyrazolo[3,4-*d*]pyrimidine (**9**) was unexpectedly weak.

Most compounds tested in this and the previous study did not bind to the ANS site. This was unexpected, as the basis for examining 6-benzyladenine binding to LBL was its apparent structural similarity to ANS. Yet 6-benzyl-adenine only weakly inhibited ANS binding at mM concentrations [10]. Some 9-benzyladenines, however, appear to competitively inhibit ANS binding. To better understand the specificity of the ANS site, three compounds containing partial structures of ANS or TNS were tested in the fluorescence assay. 2-Naphthylamine, an analog of TNS, weakly enhanced ANS fluorescence ($K_{act} > 10^{-3}$ M), presumably by binding to the adenine site. 1-Naphthylamine, a partial ANS structure, weakly inhibited ANS fluorescence ($K_{inhibition} > 10^{-3}$ M), possibly as a competitive inhibitor. In contrast to the weak binding of naphthylamines, diphenylamine, a structure contained both in ANS and TNS, bound well to the adenine site

($K_{act} = 1.2 \times 10^{-4}$ M) and enhanced ANS binding. This suggests that TNS interacts with the adenine site through the 2-toluidinylamine and that the naphthalene rings are not accommodated well in the adenine site. The failure of ANS to bind to the adenine site may result from the proximity of the 8-sulphonate to the 1-phenylamine, which may sterically block entry of the ring into the adenine site. This is also consistent with the previous finding that *N*-phenyl-1-naphthylamine binds to LBL with high affinity [9].

Our results indicate a high degree of specificity for adenine binding by LBL. Minor modifications at positions 1, 3 and 7 can block binding, suggesting specific interactions with these nitrogens. The 6-amino group is absolutely required for binding. Whereas modifications at positions 2 and 8 are tolerated, and often increased binding affinity, they also altered the allosteric interactions with the ANS site. Thus almost any modification of the adenine structure alters its interactions with LBL.

It is interesting that a free N^1 is required for both cytokinin activity [22] and LBL binding and that 2-substituted adenines are active as cytokinins and bind to LBL. 2-Methylthio-derivatives of cytokinins have also been isolated from plants [21]. In contrast, adenine is not generally active as a cytokinin [21, 22] but is bound to LBL better than most cytokinins. The similarities between LBL and cytokinin receptors could be a coincidence, whereas preferential binding of adenine may indicate a function of LBL in adenine biochemistry independent of the cytokinins.

The fine specificity of adenine binding to other legume lectins remains to be determined. Adenine binds with high affinity to *Phaseolus vulgaris*-E_4 isolectin, *Dolichos biflorus* seed lectin and the cross reacting material to the seed lectin isolated from leaves and stems of *Dolichos biflorus* [10, 23]. Further study of these lectins may be useful for determining the significance of adenine binding.

At present the roles of both adenine binding and carbohydrate binding in the biology of lectins in their respective plants is unknown. LBL is an *N*-acetylgalactosamine binding lectin; yet *N*-acetylgalactosamine is an uncommon sugar in plants [24, 25]. Adenine and related metabolites are present in legumes and should be considered in efforts to determine the function of legume seed lectins.

EXPERIMENTAL

Materials. LBL was isolated from lima beans cv. Thorogreen by affinity chromatography on Synsorb A [19]. ANS (Eastman) was recrystallized from hot H_2O before use. The adenine analogs **1**–**24** (Table 1) were prepared by previously reported literature methods. Compounds **25**–**31** were prepared by procedures developed in our laboratory [20].

Fluorescence titrations. Interactions between LBL and adenine analogs were detected by perturbation of ANS binding using the previously described assay [10]. All binding constants were determined with 0.2–0.5 mg/ml LBL in NaPi buffered saline (0.1 M NaPi, 0.15 M NaCl, 0.1 mM $CaCl_2$, $MgCl_2$, $MnCl_2$, 0.01% NaN_3, pH 6.8) thermostatted at 25°. An SLM 8000 spectrofluorometer was used in ratio mode with a 1 nm excitation band width and a 4 nm emission band width. Small vols of ligand (less than 5% v/v) were added using a μl syringe to LBL containing 1×10^{-4} M ANS. Fluorescence measurements were taken following mixing and equilibration for at least 1 min after each addition. Control titrations were done in the absence of LBL to allow correction for fluorescence quenching due to the added ligand. Net fluorescence enhancement or quenching was further corrected for dilution due to added ligand.

Equilibrium dialysis. [^{14}C]-Adenine (ICN) was equilibrated with LBL in microdialysis cells (Technilab Instruments, Inc., Pequannock, NJ) for 24 hr at 25°. The concn of adenine in each chamber was determined by scintillation counting.

Acknowledgements—This work was supported by PHS Grant CA-28381, GM29470 and Training Grant 5-T32 GM-07767, and in part by funds from the Filariasis components of the UNDP/World Bank/WHO special program for Research and Training in Tropical Diseases (I.D. 840124 and 840398).

REFERENCES

1. Boyd, W. C. and Reguera, R. M. (1949) *J. Immunol.* **62**, 333.
2. Morgan, W. T. J. and Watkins, W. M. (1953) *Br. J. Exp. Pathol.* **34**, 94.
3. Roberts, D. D. and Goldstein, I. J. (1984) *J. Biol. Chem.* **259**, 903.
4. Galbraith, W. and Goldstein, I. J. (1972) *Biochemistry* **11**, 3976.
5. Gould, N. R. and Scheinberg, S. L. (1970) *Arch. Biochem. Biophys.* **137**, 1.
6. Roberts, D. D. and Goldstein, I. J. (1984) *Arch. Biochem. Biophys.* **230**, 316.
7. Pandolfino, E. R. and Magnuson, J. A. (1980) *J. Biol. Chem.* **255**, 870.
8. Galbraith, W. and Goldstein, I. J. (1970) *FEBS Letters* **9**, 197.
9. Roberts, D. D. and Goldstein, I. J. (1982) *J. Biol. Chem.* **257**, 11274.
10. Roberts, D. D. and Goldstein, I. J. (1983) *J. Biol. Chem.* **258**, 13820.
11. Yang, D. C. H., Gall, W. E. and Edelman, G. M. (1974) *J. Biol. Chem.* **249**, 7018.
12. Houston, L. L. (1980) *Biochem. Biophys. Res. Commun.* **92**, 319.
13. Chatelain, C., Oustrin, J. and Rouge, P. (1982) *Ann. Pharm. Fr.* **40**, 473.
14. Roberts, D. D. and Goldstein, I. J. (1983) *Arch. Biochem. Biophys.* **224**, 479.
15. Hardman, K. D. and Ainsworth, C. F. (1973) *Biochemistry* **12**, 4442.
16. Cunningham, B. A., Hemperly, J. J., Hopp, T. P. and Edelman, G. M. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3218.
17. Foriers, A., Lebrun, E., Van Rapenbusch, R., de Neve, R. and Strosberg, A. D. (1981) *J. Biol. Chem.* **256**, 5550.
18. Kouchalakos, R. N., Bates, O. J., Bradshaw, R. A. and Hapner, K. D. (1984) *Biochemistry* **23**, 1824.
19. Roberts, D. D., Etzler, M. E. and Goldstein, I. J. (1982) *J. Biol. Chem.* **257**, 9198.
20. Arjunan, P. and Townsend, L. B. (1986) *J. Med. Chem.* (submitted).
21. Leonard, N. J. (1974) *Rec. Adv. Phytochem.* **7**, 21.
22. Skoog, F., Hamzi, H. Q., Szweykowska, A. M., Leonard, N. J., Carraway, K. L., Fujii, T., Helgeson, J. P. and Loeppky, R. N. (1967) *Phytochemistry* **6**, 1169.
23. Roberts, D. D. and Goldstein, I. J. (1983) in *Chemical Taxonomy, Molecular Biology, and Function of Plant Lectins* (Etzler, M. E. and Goldstein, I. J., eds) pp. 131–141. Alan R. Liss, New York.
24. Jennings, A. C. (1978) *J. Sci. Food Agric.* **29**, 915.
25. Wold, J. K. and Hillestad, A. (1978) *Phytochemistry* **15**, 325.