added to the bacteria promoted a more uniform flow pattern.

For good sample duplication the automated system must be kept clean. Half-hour rinsing with 0.1% Brij solution kept coils clean for several days. Skewed peaks and erratic base lines, however, indicate a need for tubing changes and acid rinses for all coils. Sample preparation was the same as reported earlier (Lowe and Hamilton, 1967).

RESULTS AND DISCUSSION

This method is readily adaptable to a wide range of nitrate concentrations by making slight modifications in the autoanalyzer system as described above. Three levels of nitrate standards were measured (0–80, 0–5, and 0.–0.05 μg/ml of NO_3^-–N) and the curve of each was drawn. A linear curve resulted at each concentration and a wide range of sample types could be used. Tobacco tissue and other plant materials high in nitrate were measured by use of the higher concentration, soil at the middle range, and samples low in nitrate, such as stream or ground water, at the lower range. In fact, a measurement of as little as 0.01 ppm of nitrate/nitrogen was detected on the analyzer. Very low samples were measured by use of the concentrated stock suspension and then interchange of the sample and bacteria flow lines. Monitoring of such low levels of nitrate will probably find wide application in present and future pollution work.

The quantitativeness of the system was measured by adding a specific amount of nitrate to tobacco samples. Table I shows the recovery values for the different samples. Cured burley tobacco was used and four leaf positions of differing concentrations were taken for measurement. All recovery values were excellent with no more than ±1% difference between samples. These values show conclusively the quantitative specificity of the nitrate reductase enzyme. Even at high nitrate levels the enzyme responded effectively and gave continuous quantitative reductions.

To determine analyzer performance, a burley tobacco sample was replicated. The data of Table II shows the precision attainable with the autoanalyzer system. Replications differed no more than 0.1 ppm with a resulting error of only 0.4%. Thus, the data of Tables I and II show that reliable results can be achieved with the procedure because of the highly specific nature of the enzyme and the precision of the autoanalyzer. Few plants will be encountered that have more interfering compounds than tobacco. However, we have used the procedure with extracts of many plant materials without any complication.

The bacteria used in this method were easily cultured, and ordinary reagent grade chemicals were used in all medium preparations. With time, growth curves were relatively uniform from one harvest to the next. The extremely high enzyme levels can be duplicated if strict anaerobiosis is maintained. Oxygen severely limits high nitrate reductase levels and allows the formation of nitrite reductase; therefore, it should always be removed from the culture and stock suspensions. The entire culturing and harvesting process involves only 1 day and can be performed by any cautious technician. The harvest procedure is a critical phase of the process because temperature, resuspensions, and time control enzyme preservation. Under normal conditions, refrigerated stock suspensions have maintained activity up to 3 weeks and enough enzyme is available from a single culture to run 1200–1600 analyses.

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Structure–Activity Relationships of Some Antifungal Indoles

Wybo H. Dekker,[1] Hendrik A. Selling,* and Jan C. Overeem

In a series of 64 substituted indoles most members were found to possess antifungal activity against *Botrytis allii* and *Cladosporium cucumerinum*, while some were also active against *Penicillium italicum* and *Aspergillus niger*. Highest activities were found for 3-phenylindole and 3-(2-methylphenyl)indole. By regression analysis, the antifungal activities were found to correlate significantly with (1) a substituent constant π, derived from TLC R_f values, (2) the square of π, and (3) the NMR chemical shift of the NH proton in Me_2SO solution.

Although indoles are well known for their biological activity in medicinal chemistry and as plant growth regulators, antifungal activity has only been reported for 3-thiocyanatoindoles (Akerstrom et al., 1970, 1971). We now have found antifungal activity to be widespread among indole derivatives and in this paper we report the results obtained with 64 representatives. In addition, we give correlations between antifungal activity of the indoles with physicochemical properties by regression analysis, using the multiparameter approach according to Hansch (1971).

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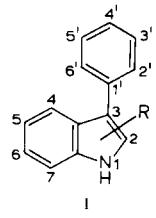
EXPERIMENTAL SECTION

Synthetic Methods. The following methods were employed for the synthesis of most of the indoles: (A) treatment of isatin or a substituted isatin with an appropriate Grignard reagent, followed by reduction of the intermediate dioxindole with $LiAlH_4$ (Bergman, 1971); (B) Fischer

Table I. Physical Constants of Indoles I



I

No.	Method of prep.	R	Mp, °C	Solvent of recryst.	Elemental anal.
1	A, B	H	88–89[g]	PhH	N
2	B	1-Me	63–64[h]	LP	N
3	B	2-Me	59–61[i]	Et_2O–LP	N
4	A	5-Me	102–103	*b*	N
5	A	7-Me	111–112	EtOH–H_2O	N
6	A	5,7-Me_2	92–93	LP	N
7	A	2′-Me	Oil	*b*	N
8	A	3′-Me	82–83	*a*	N
9	A	4′-Me	105–107	*a*	N
10	B	2,2′-Me_2	Oil	*b*	N
11	A	2′,6′-Me_2	120–121	MeOH–H_2O	N
12	A	2′,4′,6′-Me_3	67.5–69.5	*b*	N
13	A	2′-Et	Oil	*b*	N
14	A	3′-*i*-Pr	Bp 180–189 (30 Pa)		C, H
15	A	3′-CF_3	111–112	*a*	N
16	A	5,7-Cl_2	83–84	LP	C, H
17	A	5-MeO	66–67	*b*	N
18	A	2′-MeO	125–128	CCl_4	N
19	A	4′-MeO	134–138[j]	PhH	N
20	A	2′-MeS	Oil	*b*	N,[e] S[e]
21	D	2′-MeS(O)	Oil	*b*	N, S[f]
22	D	2′-$MeSO_2$	Oil	*b*	N, S
23	D	2′-NO_2	119–120[k]	EtOH–H_2O	N
24	B	2-COOEt-2′-NO_2	142–143	EtOH–H_2O	N
25	D	2-COOH-2′-NO_2	275[k]	EtOH–H_2O	N
26	D	2′-NH_2	Oil[k]		N[c,e]
27	D	1-COMe	137–138[l]	EtOH	N
28	D	2-COPh	203–205[m]	EtOH	N
29	D	1-Ph	102–103[n]	EtOH	N
30	D	1-PhNHCO	117–118	CCl_4	N
31	D	1-MeNHCS	125–127	MeOH–H_2O	N
32	D	1-Cl_3CS	71–74	*b*	N, Cl[d]
33	A	2′,3′-Benzo	~80	*b*	N
34	A	3′,4′-Benzo	133–134	PhH–LP	N
35	D	2-Ph	124–125[o]	MeOH	N

[a] Sublimated in vacuo. [b] Purified by column chromatography. [c] Hydrochloride. [d–f] Elemental analysis found: [d] 0.44% higher; [e] 0.24% lower; [f] 0.29% lower than calculated. [g] Fischer and Schmidt (1888). [h] Julia and Lenzi (1962). [i] Trenkler (1888). [j] Bruce (1959). [k] Kermack and Slater (1928). [l] Bruce and Sutcliffe (1957). [m] Jones and Suarez (1972). [n] Dolby and Lord (1969). [o] Japp and Murray (1894).

indole synthesis (Brown, 1972); (C) indoles carrying CH_2SR substituents in position 3 were synthesized from gramine and the corresponding thiol (Licary and Dougherty, 1954; Poppelsdorf and Holt, 1954; Baciocchi and Schiroli, 1968); (D) miscellaneous methods; for compounds prepared by known methods references are given in Tables I and II.

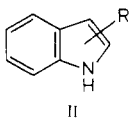
The others were prepared as follows. 1-[*N*-Methyl(thiocarbamoyl)]-3-phenylindole (**31**), 1-(*N*-phenylcarbamoyl)-3-phenylindole (**30**), and 3-phenyl-1-trichloromethylsulfenylindole (**32**) were prepared by the addition of 1-lithio-3-phenylindole in tetrahydrofuran to a solution of methyl isothiocyanate, phenylcarbamoyl chloride, and trichloromethanesulfenyl chloride, respectively, in the same solvent. The products were purified by column chromatography.

3-[2-(Methylsulfinyl)phenyl]indole (**21**) and 3-[2-(methylsulfonyl)phenyl]indole (**22**) were prepared by oxidation of the corresponding sulfide **20** (4.8 g, 20 mmol) with 4% hydrogen peroxide in acetic acid (60 mmol) for 2 hr at 80–85°. The two products were separated and purified by chromatography, yielding 280 mg of each.

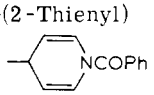
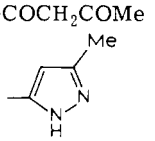
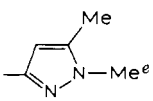
Physical Methods. Melting points were determined with a melting point microscope and are uncorrected.

Elemental analyses performed on the compounds are indicated in Tables I and II by the symbols of the elements analyzed, which further implies that the values found deviate less than 0.2% from the calculated values. Higher discrepancies are mentioned in a footnote. For all compounds presented in Tables I and II ir, uv, and ^1H NMR spectra were run, which were found to be in accordance with the proposed structures. These spectroscopic data have been deposited as supplementary material on microfilm (see

Table II. Physical Constants of Indoles II



II

No.	Method of prep.	R	Mp, °C	Solvent of recryst.	Elemental anal.
36	*d*	H			
37	*d*	3-Me			
38	A	3-Et	34–36[f]	*a*	N
39	A	3-Bu	Oil[c,g]	*b*	N
40	A	3-Hex	Oil	*b*	N
41	B	2-Me-3-pent-5-NO_2	108–110[h]	EtOH–H_2O	N
42	B	3-Hex-5-NO_2	81.5–82.5	MeOH–H_2O	N
43	A	3-CH_2Ph	106–107[f]	*a*	N
44	*d*	3-CH_2NMe_2			
45	C	3-CH_2SPr	45–46[i]	LP	N
46	C	3-CH_2SBu	39–42[i]	LP	S
47	C	3-CH_2SCH_2Ph	69–72[j]	LP	S
48	C	3-CH_2SPh	79–80[j]	LP	S
49	C	3-CH_2SPh-4′-Me	105–107[k]	LP	S
50	C	3-CH_2SPh-4′-Cl	82–83.5	LP	S
51	*d*	2-Ph			
52	*d*	2,3-Benzo			
53	A	3-(2-Thienyl)	97–98[f]	*a*	C, H, N
54	D	3- 	127–130[l]	EtOH	N
55	D	3-(4-Pyridyl)	219–220[l]	MeOH	N
56	D	3-(2-Quinolyl)	192–193[m]	EtOH	N
57	B	2-Me-3-SPh	130–131[n]	Ligroin	N
58	D	3-N=N-Ph	133–134[o]	EtOH–H_2O	N
59	D	3-C(CN)=C(CN)$_2$	265–270 dec[p]	HOAc	C, H, N
60	D	3-COMe	191–192[g]	PhH	N
61	D	3-$COCH_2$COMe	145–148[g]	PhH	N
62	D	1-$COCH_2$COMe	95–96[g]	EtOH	N
63	D	3- 	224[g]	EtOH	N
64	D	3- 	160	EtOH	N

[a] Sublimated in vacuo. [b] Purified by column chromatography. [c] Picrate. [d] Obtained from commercial source. [e] This methyl group may well be located on the other N atom. [f] Bergman (1971). [g] Bergman (1968). [h] Frasca (1962). [i] Licary and Dougherty (1954). [j] Poppelsdorf and Holt (1954). [k] Baciocchi and Schiroli (1968). [l] Deubel et al. (1971). [m] Hamana and Kumadaki (1967). [n] Wieland and Rühl (1963). [o] Binks and Ridd (1957). [p] Sansen et al. (1958).

paragraph at end of paper).

The chemical shifts of the indole–NH protons used in the regression analyses were all determined on a Varian T-60 spectrometer using 4 ± 0.2% solutions in nondeuterated dimethyl sulfoxide (dried on mol-sieve 4A). It was verified that neither halving or doubling the concentration nor the presence of up to 20% of water affected the chemical shift measurably. If the identity of the NH absorption was not unequivocal, due to other nearby absorptions, it was verified by the addition of D_2O, resulting in a lower intensity for the NH absorption. The NH chemical shifts were measured relative to the low-field ^{13}C satellite of dimethyl sulfoxide and later corrected to give the shift relative to tetramethylsilane.

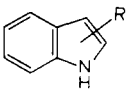
The π values used in the regression analyses were determined by the reversed phase thin-layer chromatographic (TLC) method, described by Boyce and Milborrow (1965).

For the elution of the plates a 50:50 v/v mixture of acetone and water was used. Each π value is the result of two independent measurements. The π values obtained from one measurement were systematically higher than those obtained from the other by 0.036 ± 0.029.

The mathematical operations for the regression analyses were performed by an Algol-60 program, written by one of the authors, using a CDC-Cyber 73-26 computer system. We will be glad to deliver copies of the program on request.

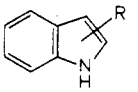
Biological Methods. Antifungal activity was determined by the roll-culture method (Pluijgers and Kaars Sijpesteijn, 1966), using a glucose mineral salts agar buffered to give a pH in the range 6.7–7.0. One drop of a spore suspension of the fungus was added to the medium in a culture bottle, each drop containing a standard amount of spores. From a stock solution of the test substance in acetone a series of dilutions was prepared and 30-mm^3 aliquots

Table III. Antifungal Activities and Physicochemical Constants of Indoles[a]



No.	R	*B. allii*	*P. italicum*	*A. niger*	*C. cucumerinum*	Δ	π	π^2
1	3-Ph	4.59	4.99	4.72	5.44	0.29	0.45	0.20
2	1-Me-3-Ph	...	...	...	...			
3	2-Me-3-Ph	4.32	...	2.62	5.02	0.10	0.53	0.28
4	5-Me-3-Ph	4.62	4.32	4.32	5.62	0.15	0.60	0.36
5	7-Me-3-Ph	3.82	...	...	4.62	0.25	0.59	0.34
6	5,7-Me_2-3-Ph	4.35	...	...	4.85	0.12	0.71	0.50
7	3-(2-MePh)	4.98	5.67	5.42	5.52	0.20	0.60	0.36
8	3-(3-MePh)	4.47	4.62	...	4.62	0.27	0.60	0.37
9	3-(4-MePh)	4.47	...	...	4.62	0.23	0.61	0.37
10	2-Me-3-(2-MePh)	4.65	...	...	4.65	−0.02	0.67	0.45
11	3-(2,6-Me_2Ph)	4.65	...	...	4.65	0.10	0.71	0.50
12	3-(2,4,6-Me_3Ph)	4.67	...	...	4.67	0.07	0.87	0.75
13	3-(2-EtPh)	4.65	...	...	5.04	0.23	0.72	0.52
14	3-(3-*i*-PrPh)	4.37	...	...	4.67	0.25	0.89	0.80
15	3-(3-CF_3Ph)	4.72	2.72	...	4.72	0.48	0.79	0.62
16	5,7-Cl_2-3-Ph	4.72	...	...	5.12	0.92	0.99	0.99
17	5-MeO-3-Ph	4.20	4.20	4.20	5.35	0.13	0.35	0.12
18	3-(2-MeOPh)	4.35	4.65	...	4.50	0.18	0.37	0.14
19	3-(4-MeOPh)	...	...	...	3.50	0.21	0.37	0.13
20	3-(2-MeSPh)	4.68	4.53	...	4.68	0.23	0.46	0.21
21	3-(2-MeSOPh)	2.71	...	...	...	0.47	−0.06	0.00
22	3-(2-$MeSO_2$Ph)	2.74	...	...	2.74	0.45	0.07	0.00
23	3-(2-NO_2Ph)	4.38	4.38	4.08	4.53	0.45	0.28	0.08
24	2-COOEt-3-(2-NO_2Ph)	...	...	...	...	1.05	0.36	0.13
25	2-COOH-3-(2-NO_2Ph)	...	...	...	...	0.90	−0.85	0.72
26	3-(2-NH_2Ph)·HCl	3.39	3.39	3.39	3.39	0.67	0.22	0.05
27	1-COMe-3-Ph	...	...	...	...			
28	2-COPh-3-Ph	2.77	...	...	...	0.95	0.74	0.54
29	1,3-Ph_2	...	...	...	...			
30	1-CONHPh-3-Ph	...	...	...	...			
31	1-CSNHMe-3-Ph	2.73	...	...	...			
32	1-$SCCl_3$-3-Ph	3.19	...	...	...			
33	3-(1-Naphthyl)	...	...	...	4.69	0.37	0.69	0.47
34	3-(2-Naphthyl)	...	...	...	...	0.40	0.73	0.53
35	2,3-Ph_2	...	...	...	...	0.50	0.87	0.76
36	H	3.22	2.92	2.92	3.22	0.00	0.00	0.00
37	3-Me	3.42	3.12	3.12	3.42	−0.35	0.17	0.03
38	3-Et	3.66	3.31	3.46	3.66	−0.35	0.36	0.13
39	3-Bu	4.24	3.54	3.54	4.24	−0.35	0.74	0.54
40	3-Hexyl	4.30	...	...	5.00	−0.35	1.12	1.25
41	2-Me-3-pentyl-5-NO_2	...	...	...	...	0.43	0.94	0.88
42	3-Hexyl-5-NO_2	...	...	...	...	0.53	1.07	1.15
43	3-CH_2Ph	4.17	...	...	4.32	−0.22	0.55	0.31
44	3-CH_2NME_2	2.51	...	...	...	−0.15	1.39	1.94
45	3-CH_2SPr	3.61	3.61	3.61	4.01	−0.20	0.52	0.27
46	3-CH_2SBu	4.04	...	...	4.04	−0.34	0.69	0.48
47	3-CH_2SCH_2Ph	4.40	...	...	4.40	−0.12	0.65	0.42
48	3-CH_2SPh	4.38	...	...	4.38	−0.10	0.58	0.34
49	3-CH_2S-(4-MePh)	3.71	...	...	4.71	−0.15	0.74	0.55
50	3-CH_2S-(4-ClPh)	4.44	...	...	5.14	−0.10	0.85	0.72
51	2-Ph	...	...	...	...	0.47	0.49	0.24
52	2,3-Benzo	...	...	...	...	0.18	0.36	0.13
53	3-(2-Thienyl)	4.30	4.30	4.60	5.60	0.35	0.41	0.17
54	3-(1-COPh-4-dihydropyridyl)	2.78	...	...	2.78	−0.12	0.46	0.21
55	3-(4-Pyridyl)	4.29	...	...	3.75	0.62	0.06	0.00
56	3-(2-Quinolyl)	...	...	...	...	0.58	0.41	0.17
57	2-Me-3-SPh	4.68	...	...	4.68	0.58	0.69	0.48
58	3-N=N−Ph	4.35	4.04	4.04	4.65	1.02	0.53	0.28
59	3-C(CN)=C(CN)$_2$	...	...	...	...	2.12	0.03	0.00
60	3-COMe	2.70	3.05	...	2.70	0.85	−0.50	0.25

Table III (*Continued*)



No.	R	*B. allii*	*P. italicum*	*A. niger*	*C. cucumerinum*	Δ	π	π^2
61	3-$COCH_2COMe$	...	...	...	...	1.00	−0.23	0.05
62	1-$COCH_2COMe$	3.45	3.30	3.30	3.61			
63	3-(5-Me-3-pyrazolyl)	...	...	...	...	0.15	−0.14	0.02
64	3-(1,5-Me_2-3-pyrazolyl)	3.48	...	...	3.63	0.47	0.04	0.00

[a] Antifungal activity is expressed as pC values (see Experimental Section). Three dots indicates that no complete inhibition occurred at the 500-ppm level.

were added to the agar.

The dilutions were chosen such as to reach a final concentration of the test compound in the agar of 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 ppm, etc. The highest concentration applied was 500 ppm.

After 3 days of incubation at 24° the bottles were investigated for the presence of growth. The minimum concentrations in parts per million of the substances, causing complete inhibition of growth, were read and recalculated in molar concentrations. pC values were obtained by conversion of the latter to their negative logarithms. The antifungal activity is established as the average of at least two independent measurements.

RESULTS AND DISCUSSION

In Table III the in vitro antifungal activities of the indole derivatives toward the four test fungi are summarized. Table III also shows the values of the empirical electronic and lipophilic parameters of the compounds, which have already been discussed in the Experimental Section.

The indoles show a marked selectivity in their action upon the different fungi: *B. allii* and *C. cucumerinum* are much more sensitive toward the indoles than *P. italicum* and *A. niger*.

Substitution at position 1 or 2 in the indole nucleus in an active compound (e.g., 3-phenylindole (1)) cancels out activity almost completely, which led us to the conclusion that the NH group plays an important role in the activity.

For the N-unsubstituted indoles regression analyses have been performed with the following quantities as the independent parameters. Δ is the NMR NH shift of the indoles in Me_2SO; since the NH group seems to be involved in antifungal action, its electronic state is probably the most proper measure for the electronic effects of the substituents. The successful correlation of biological activity with OH chemical shifts in phenols (Cronenberger et al., 1969) and the NH chemical shifts in sulfonamide drugs (Kakeya et al., 1970) has been reported in the literature. π is the difference between the logarithms of the partition coefficients of a substituted indole derivative and the parent compound (indole), which has been shown (Leo et al., 1971) to be linearly correlated with the sum of the lipophilic substituent constants of all substituents. π^2 is introduced in order to account for possible second degree dependencies in the activity vs. π relationship (Penniston et al., 1969).

It should be noted that the results of the regression analyses must be interpreted with care, since the method applied is only statistically sound if the parameters used are really independent, which is not the case for π and π^2. The coefficients of the regression equations for the four fungi using all 1-unsubstituted indole derivatives have been compiled in the first column of Table IV.

In all cases, except *A. niger*, the contribution of the π term appears to be quite significant. In all cases, too, its contribution is more important than that of the other terms. For *B. allii* there is also a highly significant contribution of π^2, indicating an optimum value for π. The contribution of the Δ term is only significant for *B. allii* and *C. cucumerinum*. In view of the small coefficients, this term is only of minor importance as compared with the π term.

Although in the regression equations for *B. allii* and *C. cucumerinum* most of the coefficients point to highly significant contributions of the corresponding terms, the correlation coefficients are too low for reliable predictions to be made about the activity of new indole derivatives. This is probably due to influences not accounted for, and which are likely to occur especially in heterogeneous sets of compounds. Therefore, regression analyses were applied on two more coherent, monosubstituted subgroups, viz. the 3-alkylindoles (**36–40** and **43–50**) and the 3-arylindoles (**1, 7–9, 11–15, 18–23, 26, 33,** and **34**). The results for the 3-alkyl-substituted indoles are summarized in the second column of Table IV.

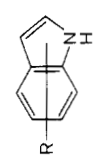
For *B. allii* and *C. cucumerinum* the correlations are far better than those obtained previously. Apparently *P. italicum* and *A. niger* give outstanding correlations too (r = 0.99 and 0.998, respectively), but since only one degree of freedom was left, the equations have almost no value from a predictive point of view. On the other hand, the coefficients clearly underscore the trends with Δ, π, and π^2 found in the other equations, although their values are not very reliable, as reflected by their low confidence levels. Again for all fungi (except for *C. cucumerinum*) a parabolic relationship with π is observed.

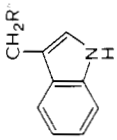
In the third column of Table IV the results of the regression analyses for the 3-arylindoles are compiled. The number of compounds of this type which are active against *A. niger* was insufficient to apply regression analysis. For the other fungi the coefficients in the regression equations were all highly significant, accounting for the acceptable correlations found. The high values for the coefficients k_2 and k_3 associated with π and π^2 in these equations indicate that activity is highly sensitive for any variation in π, while being confined to a narrow range of π values. However, these coefficients are improbably high in the second equation. In fact, it appears that, due to the small number of compounds available for this regression equation, the steep dependency of the activity on π is largely determined by only one compound. Therefore, a predictive value should not be attributed to this equation.

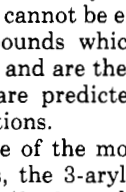
The Δ term is absent or insignificant in all three equations. It seems therefore that electronic influences do not play an important role within this series. The rather great diversity of the substituents and thence the large spread in Δ values may corroborate this statement.

A remarkable phenomenon about the regression analyses in the last two series is the conformity of π_o: 0.69 ± 0.23,

Table IV. Equations Correlating Antifungal Activity with Physicochemical Constants[a]







	k_1	k_2	k_3	k_4	r	ν	π_o	k_1	k_2	k_3	k_4	r	ν	π_o	k_1	k_2	k_3	k_4	r	ν	π_o
B. allii	0.61	2.78	−1.85	3.23	0.74	38	0.75		3.54	−2.58	2.96	0.81	10	0.69	−0.58	5.40	−4.08	3.05	0.95	11	0.66
SE	0.22	0.41	0.37	0.16			0.19		0.86	0.59	0.30			0.23	0.54	0.85	0.87	0.33			0.17
% conf. level	99.2	100.0	100.0	100.0					99.8	99.9	100.0				68.7	100.0	99.9	100.0			
P. italicum	0.58	1.84	−1.91	3.57	0.51	14	0.48	0.59	2.40	−1.74	2.93	0.99	1	0.69		20.97	−21.77		0.89	6	0.48
SE	0.50	0.85	1.43	0.35			0.42	0.31	0.52	0.61	0.07			0.29		1.22	1.99				0.16
% conf. level	72.9	95.3	79.6	100.0				69.5	86.5	78.5	98.6					100.0	100.0				
A. niger	0.66	1.56		3.12	0.52	11		0.50	2.74	−2.24	2.91	0.998	1	0.61							
SE	0.49	1.00		0.46				0.20	0.33	0.39	0.04			0.14							
% conf. level	79.4	85.6		100.0				76.2	92.4	89.1	99.1										
C. cucumerinum	0.55	2.53	−0.91	3.33	0.71	38	1.39	1.42	1.95		3.39	0.97	9			8.54	−6.54	2.14	0.83	13	0.65
SE	0.27	0.56	0.60	0.20			0.97	0.40	0.17		0.12					2.02	1.95	0.48			0.25
% conf. level	95.3	99.9	86.5	100.0				99.4	100.0		100.0					99.9	99.5	99.9			

[a] $pC = k_1\Delta + k_2\pi + k_3\pi^2 + k_4$. r = correlation coefficient. π_o = value of π for optimum activity. ν = degrees of freedom. [b] Including indole.

0.69 ± 0.29, 0.61 ± 0.14, 0.66 ± 0.17, 0.48 ± 0.16, and 0.65 ± 0.25, respectively. Assuming that the compounds will cross one or more lipoprotein membranes in the fungal cell before reaching their site of action, it could mean that this site of action is similar for the test fungi in both series.

But still some questions remain. For example, the relative sensitivity of *P. italicum* and *A. niger* toward the indoles cannot be explained on this basis. Furthermore, many compounds which are completely inactive against these fungi and are therefore not included in the regression analysis are predicted to be highly active by the regression equations.

One of the most surprising features occurs in the third series, the 3-arylindoles. Three isomers have been tested, viz. 3-(2-, 3-, and 4-tolyl)indole, compounds **7, 8,** and **9,** respectively. Whereas compound **7** exhibits an outstanding activity against all four fungi, compound **8** is inactive against *A. niger,* but is still performing well against the other fungi. Compound **9,** predicted to be an active compound against *P. italicum* from the regression equation by virtue of almost the same values for the parameters as its isomers, is not toxic to this fungus, nor to *A. niger.* The same reasoning applies to 3-(2- and 4-methoxyphenyl)indole, compounds **18** and **19.**

These effects may indicate that steric interactions play an important part. However, we have not been able to develop a rational model accounting for these effects.

Taking care of the proper restrictions it may be stated that for the design of new active compounds, π should be chosen preferably in the range 0.6–0.7; electronic influences are of minor importance while steric factors, although probably present, are not predictable.

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Supplementary Material Available. A listing of ir, NMR, and uv spectroscopic data will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journal Department, American Chemical Society, 1155 16th St., N.W., Washington, D.C. 20036. Remit check or money order for $4.00 for photocopy or $2.50 for microfiche, referring to code number JAFC-75-785.

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Differences in the Amount and Range of Volatile Carbonyl Compounds Formed by Lipoxygenase Isoenzymes from Soybeans

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The lipoxygenases from soybeans, L-1 (optimum pH 9.0), L-2 (pH 6.5), and L-3 (pH 6.5), were incubated with linoleic and with linolenic acid. The carbonyl compounds formed were isolated and analyzed as 2,4-dinitrophenylhydrazones. The experiments showed that L-2 and L-3 form significantly more carbonyl compounds than the alkaline enzyme L-1. From reactions with linolenic acid as substrate the relative amounts of carbonyl compounds which were formed during the catalysis were estimated (mole percent). L-3 formed propanal (41), 2-*trans*-pentenal (11), 2-*trans*-hexenal (9), 2-*trans*,6-*cis*-nonadienal (2.5), 2-*trans*,4-*cis*-heptadienal (20), 3,5-octadien-2-one (8), and 2,4,6-nonatrienal (8.5). In the experiment with the other neutral lipoxygenase a similar range of carbonyl compounds was identified. In contrast, only 2-*trans*-hexenal (77), propanal (18), and 2-*trans*-pentenal (5) arise during the incubation of linolenic acid with L-1.

Various volatile carbonyl compounds which arise from enzymatic oxidative breakdown of unsaturated fatty acids are detected in the aroma of fruits and vegetables (Drawert et al., 1966, 1973; Grosch and Schwarz, 1971). These volatile aldehydes and ketones only occur in plants in extremely small concentrations, more of them being formed if the cell has broken so that oxygen can penetrate the tissue.

To get an insight into the formation of volatile carbonyl compounds we have begun to study model systems containing an unsaturated fatty acid and a factor which could possibly promote lipid peroxidation in plant tissues. In a first paper (Grosch et al., 1974) we have compared the patterns of volatile carbonyl compounds arising from linolenic acid by autoxidation (accelerated by haemoglobin), by singlet oxygen and in the presence of lipoxygenase. In these experiments we have used a purified lipoxygenase isoenzyme from peas (pH optimum 6.5). However, not only neutral lipoxygenases occur in plants. For example, soy beans and peanuts contain additionally an alkaline isoenzyme (pH optimum 8.5–9.0) (Christopher et al., 1970, 1972a; Dillard et al., 1960).

Studies on the lipoxygenase isoenzymes of soybeans have shown that they differ not only in pH optimum but also in their power to co-oxidize carotenoids (Weber et al., 1974) and in substrate (Christopher et al., 1970, 1972a) and peroxidation specificities (Christopher et al., 1972b; Leu, 1974; Roza and Francke, 1973). In view of the formation of volatile carbonyl compounds it is of interest to investigate the neutral and alkaline lipoxygenase isoenzymes from soybeans in relation to their capacity for promoting the breakdown of unsaturated fatty acids to such flavor compounds. This article deals with the observed qualitative and quantitative differences.

EXPERIMENTAL SECTION

Materials and Reagents. The following materials were used: soybeans (Harburger Ölwerke, Brinckmann and Mergell); linolenic acid (>99%, Nu Chek Prep); Tween 20 and 80 (Schuchardt); piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes buffer, Sigma); Al_2O_3 neutral (Woelm); Seasorb 43 (Fisher Scientific); 2,4-dinitrophenylhydrazine (DNPH; Merck), recrystallized from benzene; *n*-heptane (Merck) was freed from carbonyl compounds as described for *n*-hexane (Grosch, 1968). The other chemicals were analytical grade.

Separation of Lipoxygenases-1, -2, and -3 from a Soy Extract. The lipoxygenase isoenzymes were separated by DEAE-cellulose chromatography as previously described (Weber et al., 1974). For clear designation of the isoenzyme an elution diagram is shown in Figure 1. The fractions containing an isoenzyme were collected and the protein content calculated from the extinction at 280 nm with $E_{1\ cm}^{1\%}$ of 14.2.

Determination of the Lipoxygenase Activity. After DEAE chromatography the collected isoenzymes were assayed at 23° at pH 6.5 (L-2 and L-3) and at pH 8.5 (L-1) using a modification of the Surrey substrate (Surrey, 1964). The assay mixture contained 1.65 m*M* linoleic acid, 0.5 μl/ml of Tween 20, 0.1 *M* sodium phosphate buffer (pH 6.5), or 0.1 *M* sodium borate buffer (pH 8.5). In 3 ml of reaction mixture one unit caused a $\Delta E_{234}^{1\ cm}$ of 1.0 between 30 and 60 sec.

Oxidation Experiments. *Fatty Acid Emulsion.* One-hundred milligrams of linoleic acid or linolenic acid was (with the addition of 5 ml of 0.001% Tween 80 and some drops of 1 *N* NaOH) dissolved in 20 ml of H_2O. The solution was diluted to 360 ml with 0.025 *M* Pipes buffer (pH 6.5) or 0.025 *M* Tris-HCl buffer (pH 8.5). The pH of the emulsion was corrected to 6.5 or to 8.5 with dilute HCl.

Incubation. Fatty acid emulsion (360 ml), pH 6.5 or 8.5, was cooled in a 1000-ml round-bottomed flask to 10° and degassed with O_2 (5 min). L-3, L-2, or L-1 (dissolved in 40 ml of 0.01 *M* sodium phosphate buffer (pH 7.0)) was added to the emulsion and the flask closed. The reaction mixture was stirred for 20 min at 10°. (The incubation was carried out at a low temperature to hinder a breakdown of hydro-

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