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FLAVONOID BIOCIDES: PHYTOALEXIN ANALOGUES FROM CONDENSED TANNINS

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Key Word Index—Condensed tannins; phytoalexins; flavonoids; antifungal activity; antibacterial activity.

Abstract—Flavonoids containing a single alkyl chain can be synthesized from condensed tannins by thiolysis with an alkyl thiol to give epicatechin-4-alkylsulphides. A number of flavonoid derivatives were made with side chains ranging from C_6 to C_{16} and tested for fungitoxic and bactericidal activities. Maximum activity was usually found for the decane derivative. Minimum inhibitory concentrations varied with the organism tested, from about 10 ppm for some rapidly growing fungi and Gram-positive bacteria, to over 500 ppm for other fungi and Gram-negative bacteria. The structural and toxicity characteristics of the epicatechin-4-alkylsulphides suggests they are acting as analogues of prenylated isoflavonoid phytoalexins.

INTRODUCTION

Phytoalexins are biocides produced by plants in response to attack by pathogens, particularly microorganisms. A wide variety of chemical classes act as phytoalexins, including terpenoids and polyacetylenic alcohols, but the most important are flavonoids. Most flavonoid phytoalexins have an isoflavonoid structure, although compounds with a regular flavonoid carbon skeleton have also been reported [1, 2]. Non-phytoalexin flavonoids have also been isolated that have antimicrobial activity [3, 4].

There has been interest in examining the structure–toxicity relationships of phytoalexins, either using the naturally occurring compound as isolated from infected plants [5–8], or synthetic analogues [6–10]. One objective of this work is to explore the phytoalexins as possible bases for new commercial pesticides. However, there is some question whether phytoalexins or their analogues have high enough toxicity to warrant commercial application. *In vitro* tests using media treated with test compounds showed reasonably good activity against pathogens, while mixed results were obtained when tested on plants [5, 11, 12]. An understanding of the structure/toxicity relationships and toxicity mechanism are necessary for further development of useful analogues.

Perrin and Cruickshank [13] suggested that the antifungal activity of the pterocarpan-type isoflavonoids is dependent on the molecule adopting a bent shape with the two aromatic rings perpendicular to each other. This view was disproved by VanEtten [7] who found no obvious relation between structure and activity. Other studies on 3-phenylcoumarins [9] and 2-phenylbenzylfurans [10] also showed no clear correlation, other than that a balance between hydro- and lipophilicity is necessary. As an example of this latter requirement, it was demonstrated that only one hydroxyl group was necessary for activity in the benzylfurans, fully methylated and polyhydroxy derivatives being inactive. Detoxification of flavonoid phytoalexins often occurs by hydroxylation or some other reaction that makes them more polar [14], again indicating the need for a balance between polar and non-polar structural features in the active compound. Two common structural features of isoflavanoid phytoalexins, that in some cases are necessary for activity, are the isoprene substituents as is found in phaseollidin and the dioxole ring of pisatin. The mode of action and toxic structural requirements of phytoalexins are, however, rather poorly understood [15].

Condensed tannins (proanthocyanidins or procyanidins) are polyflavonoids found in a wide range of plant tissues. A typical bark procyanidin with epicatechin (upper) units attached to a catechin terminal unit is shown as structure **1**. Derivatives of the upper monomer units can be made by depolymerization under either acidic or basic conditions [16, 17]. Acidic cleavage with thiols is a common analytical technique to determine the monomeric constituents of a condensed tannin. Sulphide derivatives of the upper units are formed (e.g. **2–6**), and the lower terminal units released as the free flavanol, in this case catechin (**8**) (Fig. 1).

RESULTS AND DISCUSSION

A series of epicatechin alkylsulphides (**2–6**) were synthesized by reacting purified loblolly pine (*Pinus taeda*) or Eastern hemlock (*Tsuga canadensis*) bark tannin with hexyl (C_6), octyl (C_8), decyl (C_{10}), dodecyl (C_{12}) or hexadecyl (C_{16}) 1-thiols. The minimum inhibitory concentration (MIC) obtained with these compounds on a number of different wood-destroying fungi and some bacteria are shown in Tables 1 and 2. Changes in MIC with respect to the chain length of the alkyl substituent on the epicatechin derivative for some of the fungi and bacteria tested are shown in Figs 2 and 3. For the fungi, it was found the toxicity peaked at a chain length of 10 carbon atoms except for *Poria placenta* for which maximum activity was seen for the eight-carbon derivative. The sensitivity to epicatechin-decylsulphide was found to be

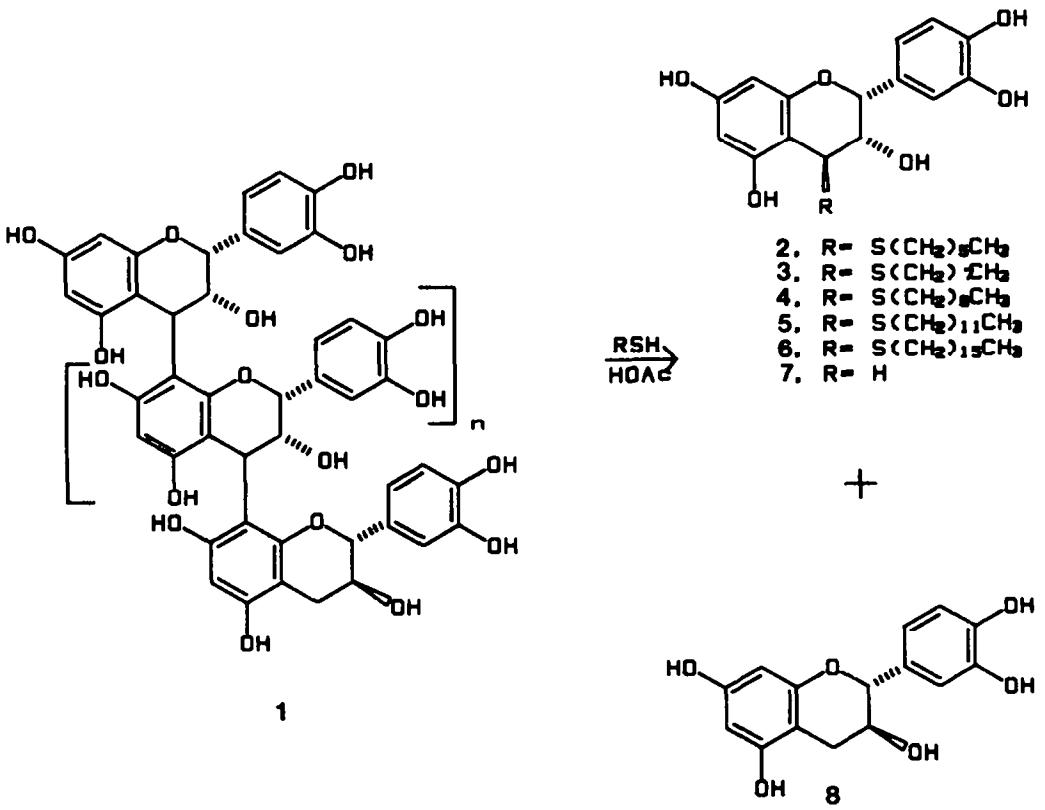

Fig. 1. Acidic thiolysis of condensed tannins.

Table 1. Minimum inhibitory concentration of epicatechin alkylsulphides on some wood-destroying fungi*

Organism	Alkylsulphide substituent (ppm) Hexyl	Octyl	Decyl	Dodecyl	Hexadecyl
Phanerochaete chrysosporium	250	100	50	1000	> 1000
Coriolus versicolor	800	250	200	> 1000	> 1000
Poria placenta	500	250	1000	> 1000	> 1000
Gloeophyllum trabeum	> 1000	> 1000	1000	> 1000	> 1000
Scytalidium lignicola	80	25	8	100	200
Lecythophora hoffmannii	800	400	300	> 1000	> 1000

*Maximum concentration tested—1000 ppm.

Table 2. Minimum inhibitory concentration of epicatechin alkylsulphides and streptomycin on some bacteria

Organism	Alkylsulphide Substituent (ppm) Hexyl	Octyl	Decyl	Dodecyl	Hexadecyl	Streptomycin
Gram-positive bacteria						
Streptococcus faciens	200	60	30	75	100	50
Bacillus cereus	80	20	5	50	75	20
Micrococcus luteus	50	40	5	50	75	8
Staphylococcus aureus	80	50	40	50	250	80
Gram-negative bacteria						
Klebsiella pneumoneae	500	> 500	> 500	> 500	> 500	8
Escherchia coli	300	500	500	> 500	> 500	5
Enterobacter cloacae	300	—	> 500	—	> 500	1
Pseudomonas aeruginosa	> 500	—	300	—	50	50

*Maximum concentration tested—500 ppm.

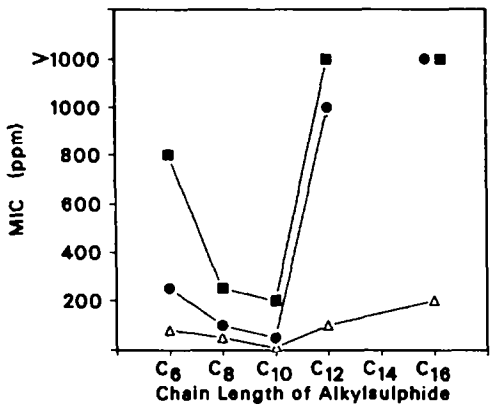

Fig. 2. Sensitivity of some fungi to epicatechin alkylsulphides: ● *Phanerochaete chrysosporium*, ■ *Coriolus versicolor*, △ *Scytalidium lignicola*.

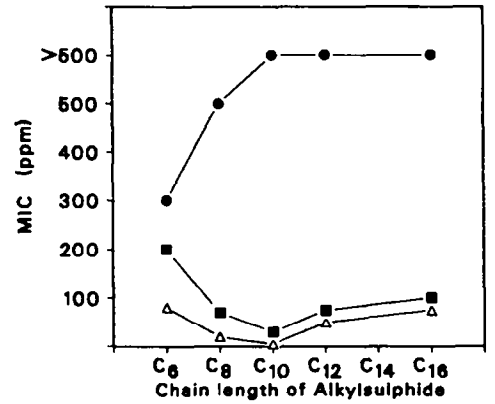

Fig. 3. Sensitivity of some bacteria to epicatechin alkylsulphides: ● *Escherichia coli*, ■ *Streptococcus faciens*, △ *Bacillus cereus*.

species-dependant, as is the case with most biocides. MICs ranged from 8 ppm for *Scytalidium lignicola* to about 1000 ppm for *Gloeophyllum trabeum*. The more rapidly growing fungi were usually more sensitive. Amongst the bacteria, the Gram-positives were much more sensitive than the Gram-negatives, again with the activity peaking at the decyl derivative. Similar selectivity against Gram-positive bacteria has been observed with phytoalexins [18, 19]. With the particular test methodology and bacterial strains used here, epicatechin-4-decylsulphide was significantly more bactericidal to Gram-positives than streptomycin, the control antibiotic used. A totally different response is seen with the Gram-negative bacteria. The smallest alkyl substituent has the greatest bactericidal activity with most Gram-negative species tested, but is still not very active. *Pseudomonas aeruginosa* showed the opposite effect, with the 16-carbon derivative having the greatest activity. The underivatized flavanol (epicatechin, 7) was tested as well and found to have no fungicidal, and very mild bactericidal, properties at the concentrations tested. No inhibition of fungal growth was observed at 1000 ppm using epicatechin, with the bacteria showing only a slight reduction in the number of colonies observed at 500 ppm.

The flavonoid phytoalexin with the closest structural resemblance to the epicatechin alkylsulphides is probably kievitone: Fig. 4 shows the structures of epicatechin-4-hexylsulphide and kievitone, with the latter drawn 'upside-down' to accentuate the similarities. The lipophilic isoprenyl side chain on kievitone and the closely related phytoalexin, wighteone, is essential for their antifungal activities. The non-prenylated analogues of these phytoalexins possess little or no activity [15, 20]. This is similar to the results described above for epicatechin and the alkylsulphide derivatives of epicatechin. The remaining important functionality of kievitone,the ketone group, is not essential for activity [8]. In fact, the highest antifungal properties were observed for the flavans rather than the flavanones. A major difference between epicatechin alkylsulphides and isoflavonoid phytoalexins is the presence of the sulphur functionality in the former. It is not known whether this grouping is necessary for biocidal activity. However, some sulphur containing, but otherwise unrelated, phytoalexins have been isolated [21].

There are a number of toxicological as well as structural similarities between epicatechin alkylsulphides and the naturally occurring phytoalexins. Both appear to have a broad-spectrum biocidal activity at comparable toxicity levels, although direct comparisons are difficult due to the wide variety of ways used to test phytoalexins. The same balance of lipo- and hydrophilicity is observed. In testing a variety of derivatives, a 10-carbon alkyl group on the epicatechin group gives the greatest activity. Derivatives with longer or shorter alkyl chains had reduced biocidal activity. For both the epicatechin derivatives and prenylated isoflavonoids, loss of the alkyl side chain greatly reduces or eliminates antifungal activity. Both are particularly active against Gram-positive bacteria, although this is a characteristic many biocides have.

EXPERIMENTAL

General. Condensed tannins were isolated as described by Karchesy and Hemingway [22] from loblolly pine (*Pinus taeda* L.) and eastern hemlock [*Tsuga canadensis* (L.) Carr.] and both used to synthesize the epicatechin alkylsulphides. ^{13}C NMR were run at 50 MHz. Because of the difficulty in removing H_2O from polyhydroxylated flavonoids, elemental analyses were obtained using the acetates. Acetylations were done using Ac_2O–pyridine, purified by prep. TLC.

Preparation of epicatechin alkylsulphides. Purified condensed tannin (5.0 g) was combined with the appropriate alkylthiol (5.0 g) in 95% EtOH (75 ml) with a catalytic amount of HOAc (0.5 g). The resulting soln was sealed under N_2 and heated at 105° for 18 hr. After evaporation, the resulting dark viscous material was triturated with hexane to remove unreacted thiol, then purified by CC over Sephadex LH-20 with 95% EtOH. The separation was monitored by TLC (silica gel and 4:1 $CHCl_3$–MeOH), visualized by spraying with vanillin–HCl. Typically, the yield was about 40%.

Numbering of carbon atoms. Conventional numbering is used for the flavonoid ring system with primed numbers indicating the B-ring. Double primes denote the alkyl substituent.

Epicatechin-4-hexylsulphide (2). ^{13}C NMR (50 MHz, Me_2CO-d_6): δ14.23 (C-10″), 23.10 (C-9″), 29.3–32.66 (C-1″–C-8″), 43.07 (C-4), 71.44 (C-3), 75.14 (C-2), 95.46, 96.57 (C-6,8), 100.05 (C-10), 115.18, 115.43 (C-2′,5′), 119.11 (C-6′), 131.83 (C-1′), 145.40 (C-

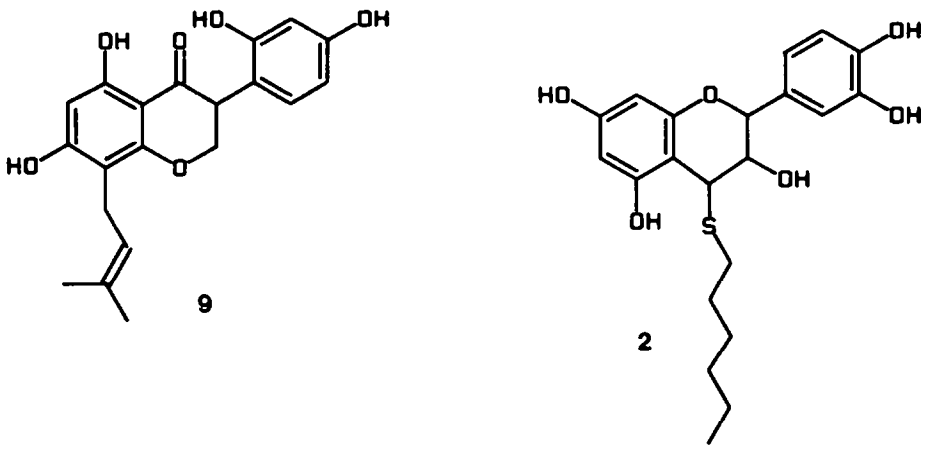

Fig. 4. Structural comparison of kievitone (9) and epicatechin-4-hexylsulphide (2).

3',4'), 158 (C-5,7,9). Found for acetate: C, 59.94; H, 6.04. $C_{31}H_{36}O_{11}S$ requires: C, 60.40; H, 5.84%.

Epicatechin-4-octylsulphide (3). ^{13}C NMR (50 MHz, Me_2CO-d_6): δ14.30 (C-10''), 23.21 (C-9''), 29.3–32.72 (C-1''–C-8''), 43.20 (C-4), 71.61 (C-3), 75.23 (C-2), 95.61, 96.70 (C-6,8), 99.79 (C-10), 115.28, 115.52 (C-2',5'), 119.20 (C-6'), 131.94 (C-1'), 145.45 (C-3',4'), 159 (C-5,7,9). Found for acetate: C, 61.92; H, 6.62. $C_{33}H_{40}O_{11}S$ requires: C, 61.50; H, 6.21%.

Epicatechin-4-decylsulphide (4). ^{13}C NMR (50 MHz, Me_2CO-d_6): δ14.05 (C-10''), 22.85 (C-9''), 29.3–32.46 (C-1''–C-8''), 42.85 (C-4), 71.10 (C-3), 74.78 (C-2), 95.11, 96.15 (C-6,8), 99.70 (C-10), 115.02, 115.52 (C-2',5'), 118.77 (C-6'), 131.36 (C-1'), 145.21 (C-3',4'), 158 (C-5,7,9). Found for acetate: C, 62.26; H, 6.70. $C_{35}H_{44}O_{11}S$ requires: C, 62.50; H, 6.55%.

Epicatechin-4-dodecylsulphide (5). ^{13}C NMR (50 MHz, Me_2CO-d_6): δ14.31 (C-10''), 23.24 (C-9''), 29.3–32.73 (C-1''–C-8''), 43.21 (C-4), 71.63 (C-3), 75.24 (C-2), 95.60, 96.70 (C-6,8), 100.17 (C-10), 115.28, 115.52 (C-2',5'), 119.21 (C-6'), 131.95 (C-1'), 145.45 (C-3',4'), 158 (C-5,7,9). Found for acetate: C, 63.26; H, 6.37. $C_{37}H_{48}O_{11}S$ requires: C, 63.43; H, 6.86%.

Epicatechin-4-hexadecylsulphide (6). ^{13}C NMR (50 MHz, Me_2CO-d_6): δ14.11 (C-10''), 23.84 (C-9''), 29.2–32.43 (C-1''–C-8''), 43.61 (C-4), 71.53 (C-3), 75.54 (C-2), 95.80, 97.70 (C-6,8), 99.37 (C-10), 114.28, 115.46 (C-2'5'), 118.81 (C-6'), 130.95 (C-1'), 144.75 (C-3',4'), 157 (C-5,7,9). Found for acetate: C, 65.06; H, 7.38. $C_{41}H_{56}O_{11}S$ requires: C, 65.08; H, 7.41%.

Test procedure for fungicidal activity. Agar plates containing the epicatechin-4-alkylsulphide to be tested were made by adding one part of an EtOH soln containing an appropriate amount of the test compound to 99 parts of molten malt extract agar. After thorough mixing, the medium was poured into 8 cm Petri dishes. When the agar had cooled and hardened, the plates were inoculated by placing a 5 mm diameter plug taken from an agar plate containing an actively growing culture of the test organism in the centre of the test plate. The diameter of the mycelium formed was measured when the culture on a control plate (containing no test biocide) had reached the perimeter of the Petri dish, or after 7 days, whichever came first. The incubation temperature was 26°. Three duplicates were run of every test compound/test organism combination and the results averaged.

Test procedure for bactericidal activity. Agar plates containing the test compounds were made as above but with brain–heart infusion agar. The plates were inoculated with a standardized suspension of bacteria cells using a calibrated 3 mm loop. After 1 day (2 days for *Micrococcus luteus*) the number of resulting bacterial cultures were counted. Three duplicates were again run and the results averaged. The incubation temperature was 35°.

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