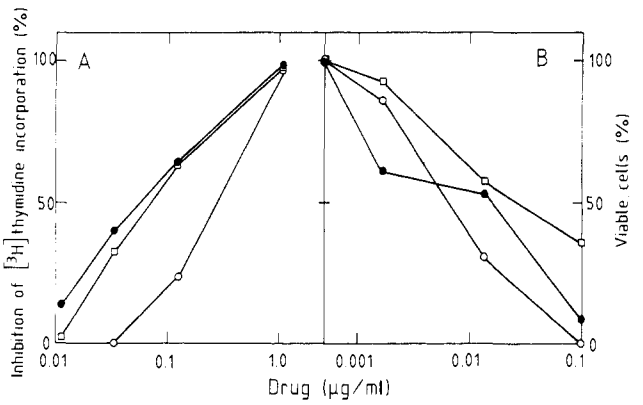

Figure 4. The pharmacological activity of the 5-fluorouridine–dextran–antibody derivative. (A) Inhibition of [*methyl*-^{3}H]thymidine incorporation as in Figure 2A except incubation with the drug and its derivatives was for 20 h. (B) Cytotoxicity as in Figure 2. (C) FU (●), FUR–dextran (○), FUR–dextran–anti-38c (□).

original activity as tested by the inhibition of DNA and RNA synthesis as well as its cytotoxic activity on the tumor cells (Figure 3C). ARA-C–dextran–anti-38 was slightly less effective on a control cell line, Daudi (human lymphoma), than a specific antibody–drug conjugate.

Pharmacological Activity of FUR–Dextran–Anti-38. The inhibition of the incorporation of [*methyl*-^{3}H]-thymidine exerted by the FUR–antibody derivative was similar to that obtained by FUR (Figure 4A) and is slightly better than that obtained by FUR–dextran without the antibody. Similar results were observed when the cytotoxic effects on the tumor cells were tested (Figure 4B). The FUR–dextran–anti-38c had a similar killing effect as FU. It should be noted that the inhibitory activity in vitro of FUR, the drug originally used for the conjugation, was much more potent than that of FU. Nevertheless, we though it right to compare the activity to FU, since we have opened the sugar ring by the periodate oxidation and used the resultant aldehyde groups for the binding. Both FUR and FU are thought to be metabolized to the same products: 5-fluorouridine monophosphate (FUMP) and 5-fluorodeoxyuridine monophosphate (FUdMp). These products exhibit the inhibitory action: FUMP by incorporation into RNA while FUdMP inhibits thymidylate synthetase. In spite of the metabolism through the same pathways, differences in their activities were noted. In vivo FUR was not active while FU was effective against the murine B leukemia 38 by prolonging survival (unpublished data). As can be seen from the data, both antibody–drug derivatives, ARA-c–dextran–anti-38 and FUR–dextran–anti-38 maintained their drug activity. ARA-c and FUR are converted biochemically in the cells to other antinucleotides which are actually the active analogues.[4] The active nucleotide of ARA-c is arabinoside cytidine triphosphate, which functions as the antagonist of the physiologic substrate deoxycitidine triphosphate and competitively inhibites DNA polymerase. The active analogues of FU are FUMP and FUdMP as mentioned above. If we assume that a similar mechanism prevails here also, thus, the drugs attached to the antibody not only have to be taken up by the cells but also have to be converted to the active analogues in situ. The penetration of drug–antibody into cells and nuclei was previously demonstrated with other anticancer agents of the tetracycline group daunomycin[11] and adriamycin.[7] The fact that the conjugates are biologically active indicates that some of the necessary biochemical conversions of antinucleotide metrabolites can be performed rapidly even on the immobilized drug. Other possibilities are that the drug conjugates operate via different mechanisms or that the drug is released slowly due to the nature of the bond. A slow release mechanism may perhaps overcome the development of resistance to antimetabolic drugs by shunt mechanisms, which is often the case with this type of drugs, particularly ARA-c. It is also assumed that a selective delivery to the site of action may result in more beneficiary chemotherapy.

Acknowledgment. The antibodies were kindly provided by Prof. J. Haimovich of the Tel Aviv University, Tel Aviv, Israel. The research was supported by a grant from Summa Corp., Albuquerque, NM.

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Substrate Specificity of Pyroglutamylaminopeptidase[1]

John T. Capecchi and Gordon Marc Loudon*

Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907. *Received March 20, 1984*

Three synthetic peptides (compounds 4–6) were tested as substrates of pyroglutamylaminopeptidase. In addition, inhibition of the hydrolysis of these substrates by compounds 8 and 9 was also examined. The enzyme does not appreciably catalyze the hydrolysis of peptides with six-membered ureido rings at the amino terminus, but it tolerates well a five-membered ureido ring at this position.

Recently in our laboratory we observed[2] that one of the products resulting from the Lossen rearrangement of α-glutamyl peptides (eq 1) is a hexahydro-2-oxo-pyrimidinyl-4-carbonyl peptide, 1. A similar five-membered derivative 2 has been observed in the Hofmann degradation of asparaginyl peptides.[3-5] The overall sequence in eq 1 is potentially valuable as a specific cleavage at glutamic acid residues, as noted by Spande et al.,[6] provided that the ureido group can be removed from the amino terminus of

(1) Abbreviations used in this paper are as follows: Pep^N, Pep^C, amino- and carboxyl-terminal portions, respectively, of a general peptide; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCU, *N*,*N*′-dicyclohexylurea; DMF, *N*,*N*-dimethylformamide; EDTA, ethylenediaminetetraacetic acid.

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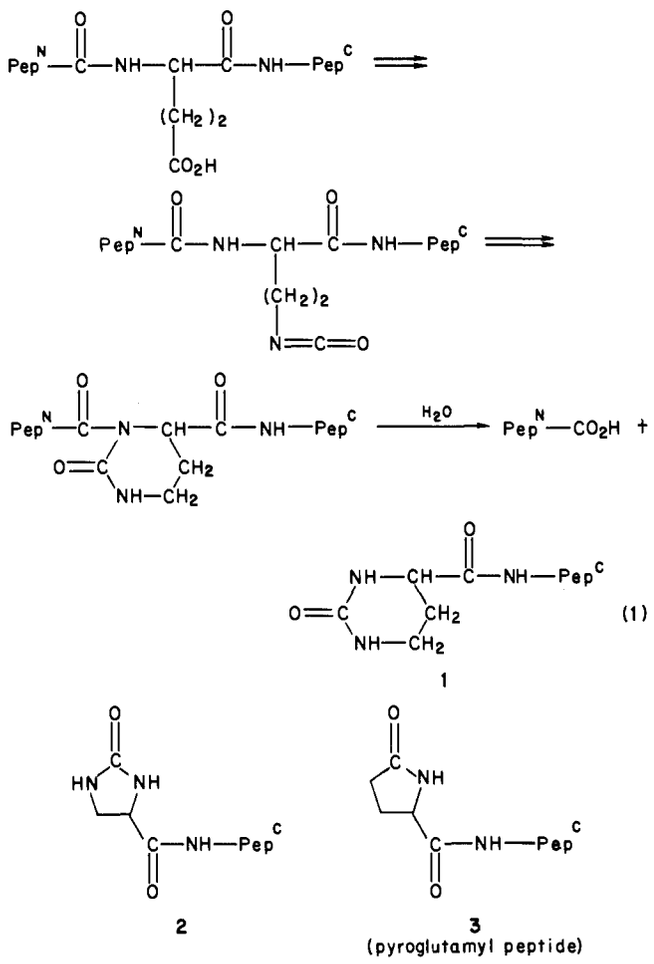


(1)

1

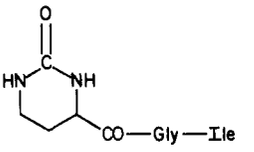
2

3
(pyroglutamyl peptide)

the peptide 1. This objective cannot be accomplished by nonenzymatic hydrolysis because of the hydrolytic stability of the six-membered cyclic urea.[7] The structural similarity of 1 to the normal substrate 3 of pyroglutamylaminopeptidase (EC 3.4.11.8), an enzyme that removes pyroglutamyl residues from peptide amino termini,[9] led us to investigate the cleavage of a peptide of form 1 by the bovine liver enzyme. We also felt that it would be of intrinsic interest to study further the substrate specificity of the enzyme using an analogous peptide incorporating the structure 2. Although there have been studies of the substrate specificity of pyroglutamylaminopeptidase,[9-11] these have involved pyroglutamyl peptides or standard peptides rather than peptides incorporating analogues of the pyroglutamyl group.

Results and Discussion

The specificity of pyroglutamylaminopeptidase was tested by subjecting the peptides 4–6 to enzymatic digestion. The results are given in Figure 1. From the figure, it can be seen that the hydrolyses of both the pyroglutamyl peptide 6 and the nitrogen analogue 5 are rapid



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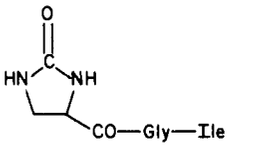

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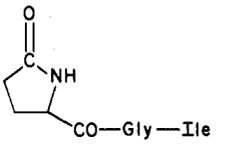

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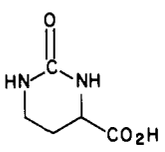
in the presence of the enzyme, but that of the six-membered nitrogen analogue 4 is slow. From these results, it appears that the enzyme can tolerate the additional nitrogen in the ring, but it is quite sensitive to the increase in ring size.

It is known that pyroglutamic acid is a competitive inhibitor of the enzyme.[12] Therefore, a preliminary study of the inhibition of the enzyme digestion of 5 and 6 by three potential heterocyclic end products of the digestion, 7–9, was carried out by digesting each substrate in the presence and absence of inhibitor. To quantitate the



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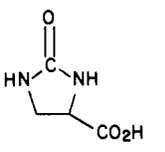

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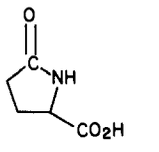

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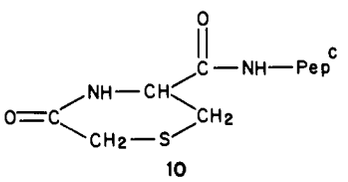
effect of the inhibitor, the enzyme digestion was allowed to proceed for 180 min in each case, the reaction was stopped, and the percent cleavage was determined by amino acid analysis (see Experimental Section). The amount of cleavage in the absence of inhibitor (see Figure 1) is given the value of 1.0 and the cleavage of substrate in the presence of inhibitor is reported as a fraction of this value in Table I. From these results it can be seen that 8 and 9 are about equally effective as inhibitors of the digestion of the corresponding peptides 5 and 6, respectively, whereas in cross-inhibition studies, 9 is a far more effective inhibitor in the hydrolysis of 5 than 8 is of the hydrolysis of 6. Of course, these preliminary studies do not allow classification of the inhibition type. Compound 7 is a poor inhibitor in all hydrolyses. These results suggest that the binding affinity of the three cyclic groups, and perhaps their analogous peptides, goes in the order 9 > 8 > 7 (progressively weaker binding).

These results show that the enzyme pyroglutamylaminopeptidase is very sensitive to the ring size of the terminating group on a potential substrate but less sensitive to the detailed structure, at least within the narrow limits tested. A six-membered ring in this position apparently cannot be tolerated by the enzyme, although the substitution of a second ureido nitrogen for carbon yields an acceptable substrate. It has been suggested that the enzyme might be used for the removal of the thio ether lactam group 10, which can form by the cyclization of an



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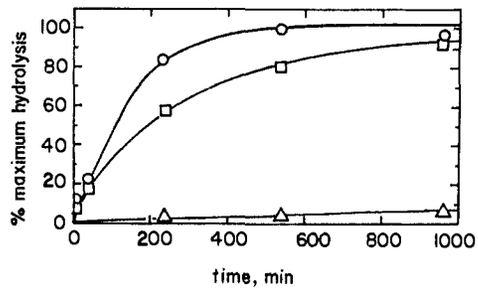

Figure 1. Hydrolysis of compounds **4** (△), **5** (□), and **6** (○) catalyzed by pyroglutamylaminopeptidase. The hydrolysis is expressed as percent of the maximum hydrolysis observed at infinite time. The amount of enzyme is 2.42 units in each experiment. The substrate concentrations are as follows: **4**, 2.49 mM; **5**, 2.93 mM; and **6**, 2.88 mM. The results are for a single series of experiments. The total extent of hydrolysis of compound **5** was 90%; that of compound **6** was 57%.

Table I. Inhibition of Pyroglutamylaminopeptidase by Product Analogues[a]

inhibitor (concn, mM)	relative hydrolysis rate of 6	relative hydrolysis rate of 7
none	1.0	1.0
9 (6.51)	0.586	0.206
9 (13.02)	0.333	0.093
8 (6.51)	0.854	0.533
8 (13.02)	0.707	0.308
7 (8.32)	0.889	0.860
7 (16.64)	0.884	0.752

[a] Pyroglutamylaminopeptidase (5.5 units) and the substrates and inhibitors at the concentrations shown were subjected to the digestion conditions described in the Experimental Section for 180 min at 37 °C. The amount of Gly-Ile released was quantitated by amino acid analysis. The results are for a single experiment.

amino-terminal (carboxymethyl)cysteine residue.[9] On the basis of the results with compounds **4** and **7**, it would appear unlikely that a compound of this type would be hydrolyzed by the enzyme.

These studies also suggest that a hexahydro-2-oxopyrimidinyl-4-carbonyl residue might be used as an effective blocking group to help minimize proteolytic digestion of peptide hormone analogues.

Experimental Section

The amino acid starting materials in all syntheses, including pyroglutamic acid, were the L enantiomers. The chemical shifts of NMR spectra in D_2O are reported relative to internal DSS (3-(trimethylsilyl)-1-propanesulfonic acid sodium salt). Chemical shifts in other solvents are reported relative to internal tetramethylsilane.

Hexahydro-2-oxopyrimidinyl-4-carbonyl-Gly-Ile (4). 2-Oxohexahydropyrimidine-4-carboxylic acid (**7**) was prepared by treatment of 2,4-diaminobutyric acid with 1,1′-carbonyldiimidazole.[2] The carboxylic acid (36 mg, 0.25 mmol) was suspended in 5 mL of CH_3CN, and DMF (5 mL) was added until dissolution. To this solution was added *N*-hydroxysuccinimide (48.7 mg, 0.42 mmol) and the solution was cooled in an ice bath. DCC (56 mg, 0.27 mmol) was added and this solution was stirred at 0 °C for 3 h and at room temperture for 5 h. The precipitate (DCU) was filtered, and the resulting solution was added dropwise with stirring to a solution of Gly-Ile (158 mg, 0.84 mmol) and $NaHCO_3$ (74 mg, 0.88 mmol). The reaction was stirred at room temperature for 12 h. The reaction mixture was filtered, and the filtrate was concentrated in vacuo and the residue was taken up in 30 mL of water. Chromatography on DEAE-Sephadex (2.5 × 40 cm, elution with a linear gradient from 1 L of H_2O to 1 L of 0.5 M acetic acid/pyridine, pH 3.9; rechromatography using 2.5 × 30 cm column, linear gradient from 1 L of H_2O to 1 L of 0.4 M acetic acid/pyridine, pH 5.0) followed by lyophilization gave **5** (49 mg, 63% yield): NMR (80 MHz, D_2O) δ 0.84–0.92 (m, 6 H), 1.0–1.5 (m, 2 H), 1.75–2.20 (m, 3 H), 3.05–3.30 (m, 2 H), 3.96 (s, 2 H), 4.05–4.30 (m, 2 H). The optical purity of this compound was not determined since it is not hydrolyzed by pyroglutamylaminopeptidase.

2-Oxoimidazolidinyl-4-carbonyl-Gly-Ile (5). This compound was prepared in the same manner as compound **4** from 2-oxoimidazolidine-4-carboxylic acid (**8**) (Aldrich), *N*-hydroxysuccinimide, and Gly-Ile. **5**: NMR (80 MHz, Me_2SO-d_6) δ 0.75–0.79 (d + t, 6 H) [1.0–1.4 (m), 1.5–1.9 (m) 3 H total], 3.1–3.65 (m, 2 H), 3.76–3.83 (d, 2 H), 3.9–4.25 (m, 2 H), 5.28 (s, 1 H), 6.52 (s, 1 H), 7.92–8.08 (m, 2 H); $[\alpha]^{20}_D$ −24.04° (*c* 2.04, methanol). This peptide was hydrolyzed to 90% of the theoretical amount by pyroglutamylaminopeptidase; this observation suggests that the peptide is of at least this optical purity at the imidazolidinone residue.

Pyroglutamyl-Gly-Ile (6). Pyroglutamic acid (**9**; 0.32 g, 2.5 mmol) was dissolved in 5 mL of pyridine and 0.63 g (1.2 equiv) of *N*-hydroxysuccinimide *O*-trifluoroacetate[13] was added. The solution was stirred at room temperature for 2.5 h and added dropwise to Gly-Ile (0.26 g, 1.38 mmol) in 5 mL of water. After addition was complete, the solution was stirred 4.5 h at room temperature. The product was chromatographed on DEAE-Sephadex (2.5 × 59 cm; elution with linear gradient from 1 L of H_2O to 1 L of 0.2 M acetic acid/pyridine, pH 3.9, followed by a gradient made from 1 L of the latter buffer and 1 L of 0.5 M acetic acid/pyridine, pH 3.9). Fractions containing product were pooled and lyophilized to give **7** (266 mg, 65%): NMR (80 MHz, $CDCl_3$/Me_2SO-d_6) δ 0.85–0.95 (d + t, 6 H), 1.0–1.5 (m, 2 H), 1.7–2,4 (m, 5 H), 3.8–3.95 (dd, 2 H), 4.0–4.2 (m, 1 H), 4.3–4.5 (m, 1 H), 7.25–7.4 (d + s, 2 H), 7.5–7.95 (br t, 1 H); $[\alpha]^{20}_D$ + 2.44° (*c* 1.5, methanol). This compound was hydrolyzed to 57% of the theoretical maximum by pyroglutamylaminopeptidase; this observation suggests that the pyroglutamyl residue is 57% optically pure.

All peptides gave satisfactory amino acid analyses and appeared to be homogeneous on thin-layer chromatography and thin-layer electrophoresis.

Assay of Pyroglutamylaminopeptidase. The enzyme (Sigma) was assayed by using a modification of the procedure of Szewczuk and Molczyk.[12] An enzyme buffer was prepared as follows. A 0.1 M Na_2HPO_4 solution (1 L) was adjusted to pH 8.0 with 0.1 M NaH_2PO_4 to give 1605 mL of solution that was made 5% (v:v) in glycerol. A deblocking buffer was prepared by making 112 mL of this solution 5 mM in dithiothreitol and 10 mM in Na_2EDTA, adjusting the solution to pH 8.0 (NaOH), and purging with N_2. The enzyme solution (0.4 mL) and deblocking buffer (0.5 mL) were combined in a test tube that was purged with Ar and sealed with a serum cap. After incubation for 3 min at 37 °C, pyroglutamyl-β-naphthylamide (Sigma, 1.78 mmol in 0.1 mL of methanol) was added, and the solution was incubated at 37 °C for 5 min. The reaction was stopped by the addition of 1 mL of a 25% trichloroacetic acid solution. To quantitate the β-naphthylamine, 1 mL of this solution was mixed with 1 mL of a 0.1% $NaNO_2$ solution. After 3 min, ammonium sulfamate (1 mL of 0.5% solution) was added to destroy excess nitrite. After 5 min, 2 mL of 0.05% *N*-1-naphthylethylenediamine dihydrochloride solution was added and the solution incubated at 37 °C for 1 h. The solution was then cooled, and the absorbance at 570 nm was read against a blank that was prepared by performing the above procedure without the pyroglutamyl-β-naphthylamide. The amount of β-naphthylamine was then determined from a standard curve prepared by using the above procedure. The unit of enzyme activity is defined as the number of nanomoles of β-naphthylamine released per minute in this assay.

Pyroglutamylaminopeptidase Digestion of Synthetic Peptides. A standard solution of the substrate peptide in the deblocking buffer and a known amount of Leu was added (as an internal standard). The ratio of substrate to Leu was determined by amino acid analysis of a hydrolyzed sample of this solution. The required amount of enzyme solution and enough deblocking buffer to bring the total volume to 0.9 mL were combined, purged with Ar, and sealed with a serum cap. This was incubated at 37 °C for 3 min and 0.1 mL of the solution of peptide and Leu was

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added. At the appropriate times, 100 μL of the enzyme digestion solution was removed, diluted with 0.5 mL of a pH 2.2 sodium citrate buffer, and frozen until analysis. A check showed that no differences occurred between samples that were stored and otherwise identical samples that were analyzed immediately. The amounts of Leu and Gly-Ile (retention time 58.3 min on the Beckman 119 amino acid analyzer) were quantitated by amino acid analysis, and the amount of hydrolysis was computed by using the previously calculated peptide/Leu ratio. For each experiment 2.42 units of enzyme were used. The substrate concentrations were as follows: **4**, 2.49 mM; **5**, 2.93 mM; and **6**, 2.88 mM.

Acknowledgment. This work was supported by grants from the National Institute of General Medical Sciences and the National Science Foundation.

Registry No. **4**, 92642-54-1; **5**, 92642-55-2; **6**, 92642-56-3; **7**, 85701-35-5; **8**, 21277-16-7; **9**; 98-79-3; DCU, 2387-23-7; Gly-Ile, 19461-38-2; pyroglutamylaminopeptidase, 9075-21-2.

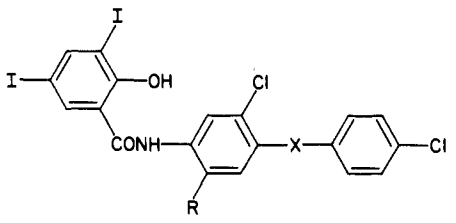
Potentiation of Fasciolicidal Agents by Benzoyl Side Chains. Synthesis of Benzoylsalicylanilides

George R. Brown,* Glynn J. Chesterson, and Gerald C. Coles

ICI Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire SK10 4TG, England. *Received May 3, 1984*

The synthesis and potent fasciolicidal activity of novel salicylanilides, with benzoyl substituents in the salicyl ring, is described. Several compounds surpassed the activity of commercially used flukicides against *Fasciola hepatica* infections in rats. Compounds **10**, **11**, and **15** were poorly active against the parasite in sheep and inactive in infected calves. It is concluded that the benzoyl substituents potentiate antiparasitic action by virtue of their electron-withdrawing properties rather than by advantageous protein binding at parasite receptor sites. Poor activity in sheep is ascribed to in vivo reduction of the carbonyl in the benzoyl group of the anilides.

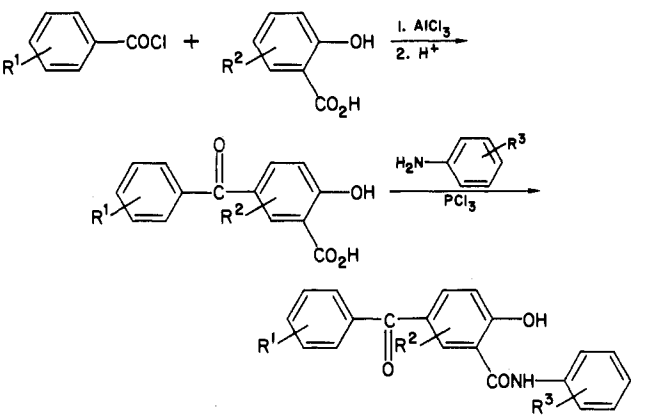
The treatment and prophylaxis of liver fluke infections in farm animals which are caused by the parasite *Fasciola hepatica* is an important objective in animal husbandry. Animals with fluke infections are in poor condition, fail to gain weight and have damaged livers. The parasite is mainly controlled in sheep by oral dosing with salicylanilide derivatives.[1] Improved fasciolicidal activity of salicylanilides has been achieved by the incorporation of an aryl side chain in the aniline moiety of the anilide, such as in rafoxanide[2] (**1**) salantel[3] (**2**), and closantel[4] (**3**). Similar substitution of a dihedral lipophilic aryl side chain in other antiparasitic agents, e.g., anthelmintic benzimidazolyl carbamates[5] and anticoccidial azauracils,[6] has also led to the potentiation of antiparasitic activity. We report the synthesis of novel salicylanilides with aryl side chains in the salicyl ring of the anilide (Tables I and II). More potent salicylanilides with a large therapeutic ratio could be dosed at high enough levels in infected animals to kill immature as well as adult liver fluke.



1, X=O; R=H
2, X=CO; R=H
3, X=CHCN; R=CH_3

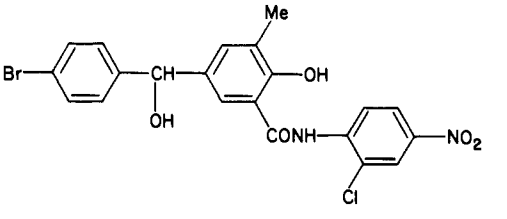
Chemistry. The anilides described in Tables I and II were prepared (Scheme I) by reaction of an appropriately substituted salicylic acid and an aniline in boiling chlorobenzene, in the presence of phosphorus trichloride. The substituted salicylic acids required to prepare compound

Scheme I



R^1=Br, Cl, CN, I, or NO_2
R^2=H, Me, or Bu^t
R^3=Br, Cl, CF_3, CN, or NO_2

6 and **7** were a gift from ICI Organics Division. The remaining acids (Table III) were synthesized by Friedel–Crafts acylation with substituted benzoyl halides into commercially available salicylic acids dissolved in nitrobenzene and in the presence of anhydrous aluminum chloride. Product isolation in high yield and purity was facilitated by the discovery that sodium salts of benzoylsalicylic acids were lipophilic and insoluble in sodium bicarbonate solutions. The preparation of the 3-*tert*-butyl acid **31** was carried out in dichloroethane below 5 °C and with excess acyl halide, because reaction in nitrobenzene caused loss of the *tert*-butyl group. Reduction of the anilide **11** with sodium borohydride afforded the benzyl alcohol **34**.



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