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DISCOVERY OF DIFLUNISAL

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1 Diflunisal (MK-647; 5-(2,4-difluorophenyl)-salicylic acid) is a new analgesic anti-inflammatory agent discovered after an extensive chemical and pharmacological study from 1962–71.

2 In the search for a superior salicylate our objectives were higher potency, better tolerance, and a longer duration of action.

3 An evaluation of many available and newly synthesized salicylates, in the granuloma and carrageenan foot oedema assays, revealed the activity-enhancing trend of a hydrophobic group—for example, phenyl, at the carbon-5 position of salicylic acid.

4 The attachment of a 5-(4-fluorophenyl) group, previously found to enhance the potency of anti-inflammatory (3,2-c)-pyrazole steroids and phenyl-α-propionic acids to acetyl salicylic acid yielded a clinical candidate flufenisal. As an analgesic, flufenisal is two times more potent than aspirin in man, but with a longer action; no distinct advantage in gastrointestinal tolerance has, however, been observed.

5 Further investigation of 5-heteroaryl salicylic acids, flufenisal congeners and their non-acylating carbonate esters identified diflunisal and 5-(1-pyrryl)-salicylic acid for subacute safety assessment. The *O*-acetyl group, commonly present in aspirin, benorylate and flufenisal, was purposely avoided in these two compounds for safety considerations.

6 Without an *O*-acetyl group, diflunisal cannot acetylate proteins and macro-molecules *in vivo* as aspirin does. In the prostaglandin synthetase assay *in vitro*, salicylic acid is much less active than aspirin. In contrast, the non-acetylated diflunisal and desacetyl flufenisal are both more active than flufenisal *in vitro*. A significant difference between aspirin and diflunisal in their biochemical mechanisms was noted.

7 On the basis of overall efficacy and tolerance data, diflunisal was finally chosen as a superior analgesic anti-inflammatory salicylate for clinical evaluation.

Introduction

Diflunisal (MK-647) is a new salicylic acid derivative with a chemical structure of 5-(2,4-difluorophenyl) salicylic acid (Figure 1a). It is the best candidate, in terms of both efficacy and safety, among approximately 500 salicylates and analogues investigated in our laboratories. The discovery of diflunisal came after an extensive collaborative effort begun in 1962. Our early chemical and pharmacological study provided a basis for its selection in 1971 as a candidate for clinical trials. More recent clinical and biochemical observations have further substantiated its potential as a chemically distinct salicylic acid derivative with much improved biological activities in man.

Following the traditional use of salicin in earlier times, synthetic salicylic acid was introduced in 1776 as a therapeutic agent. By 1889, chemist Felix Hofmann discovered that the *O*-acetyl derivative of salicylic acid, aspirin (Figure 1b), was more effective in relieving the discomfort of his arthritic parent. Since then, aspirin has become the most important and widely used pain reliever (Smith & Smith, 1966). Despite its modest anti-inflammatory/analgesic activities in animal assays (for example, 1/20–1/200 times indomethacin), in the clinic aspirin at 4–6 g/d is often considered to be as efficacious as several 'more potent' new anti-inflammatory agents. Various side-effects of aspirin, such as gastrointestinal irritation, prolonged bleeding time, tinnitus, nephrotoxicity, and so on, are well documented. Benefiting from the perspective gained in many years, clinicians have generally assumed a more tolerant view regarding the risks of salicylates. But obviously, any improved derivatives would be of considerable interest medically.

The search for a superior salicylate analogue has been an attractive yet highly elusive goal for many years. In general, the objectives are three-fold: higher

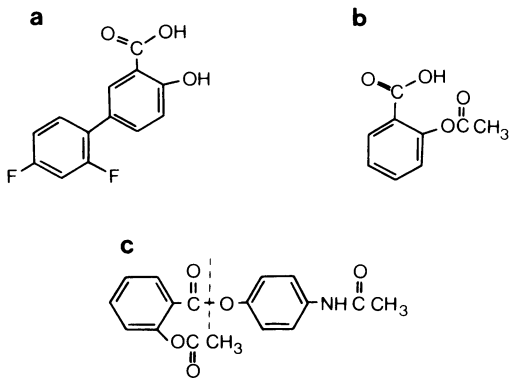


Figure 1 (a) Diflunisal; (b) aspirin; (c) benorylate.

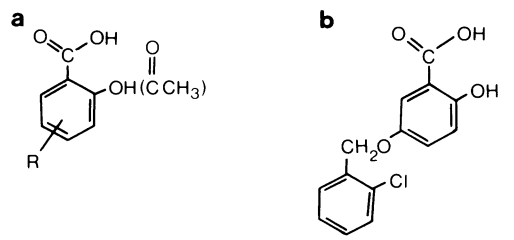
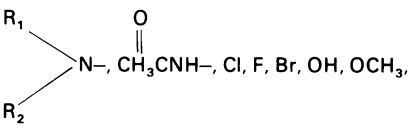


Figure 2 (a) Various substituted salicylates (R = alkyl,



and so on. (b) 5-*O*-Chlorobenzyloxy salicylic acid.

potency, better tolerance and, preferably, a longer duration of action (Shen, 1972). Numerous attempts to search for such an agent have been made, but none were really successful (Adams & Cobb, 1967). Indeed, there was a prevailing feeling that aspirin was probably unique in its class, defying further chemical modifications. More attention was then devoted to various pharmaceutical or chemical formulations, such as salts, anti-acid combinations and enteric coatings. An ester conjugate of aspirin and another mild analgesic, acetaminophen, has recently been introduced as benorylate (Figure 1c). The conjugate is readily hydrolyzed *in vivo* into its two components (Beales *et al.*, 1972).

Our laboratory study of new salicylic acid derivatives was initiated in 1962 after the completion of the indomethacin series (Shen *et al.*, 1963). It was hoped that with the newly acquired biological and chemical experiences, it might be advantageous to reassess the potential of salicylate derivatives. A number of salicylates already available and specially synthesized analogues, were evaluated in the cotton pellet granuloma, carrageenin foot oedema and yeast-induced hyperaesthesia assays in rats. At that time, these animal models seemed to be highly responsive to the anti-inflammatory and analgesic actions of non-steroidal drugs (Winter *et al.*, 1963). Selected active compounds were further evaluated in both the gastric haemorrhage and intestinal perforation assays (Brodie *et al.*, 1970).

Structure–activity relationship

Progress in our extended synthetic study was clearly marked by three stages. In the first phase, the structure–activity relationship of various substituted salicylates (Figure 2a) described by previous investigators were readily confirmed in our assays. For instance, replacement of the acetoxy group in aspirin by an alkoxy group or other substituents and derivatization of the carboxy group are generally detrimental. Ring substitution of salicylic acid with halogens or alkyl, amino, dimethylamino, acetamido, haloalkyls, hydroxy, methoxy, and other groups (Figure 2a) either reduces activity or increases both potency and toxicity in a non-specific manner. These generalizations are applicable to many newly synthesized analogues as well. Interestingly, among a number of gentisic acid derivatives prepared, 5-*o*-chlorobenzyloxy salicylic acid (Figure 2b) was found to possess fibrinolytic activity *in vitro*, about 50 times that of salicylic acid (Von Kaulla *et al.*, 1967). Subsequently, our synthetic strategy was to keep salicylic acid as the basic moiety; and several substituents previously found to be activity-enhancing in other non-steroids, such as fluoro, trifluoromethyl, phenyl and cyclohexyl groups, were systematically introduced for evaluation at the remaining 3,4,5 and 6 positions of the phenyl ring. A group of 4-phenyl salicylic acid (Figure 3a) and its esters, the amides, have been claimed to have anti-inflammatory activities. In our assays, 5-phenyl salicylic acid (Figure 3b) and its 4-chloro derivative (Figure 3c) were found to be more potent than the 4-phenyl isomer.

Our attempt to establish a more quantitative structure–activity relationship in these salicylates was hampered by the low order of their biological activities, which were very sensitive to experimental variations. As the overall physicochemical and pharmacodynamic properties of a small molecule like salicylic acid are easily influenced by multiple substitutions, in contrast to our experience with corticosteroids and indomethacin analogues, the potency enhancement of different substituents were not

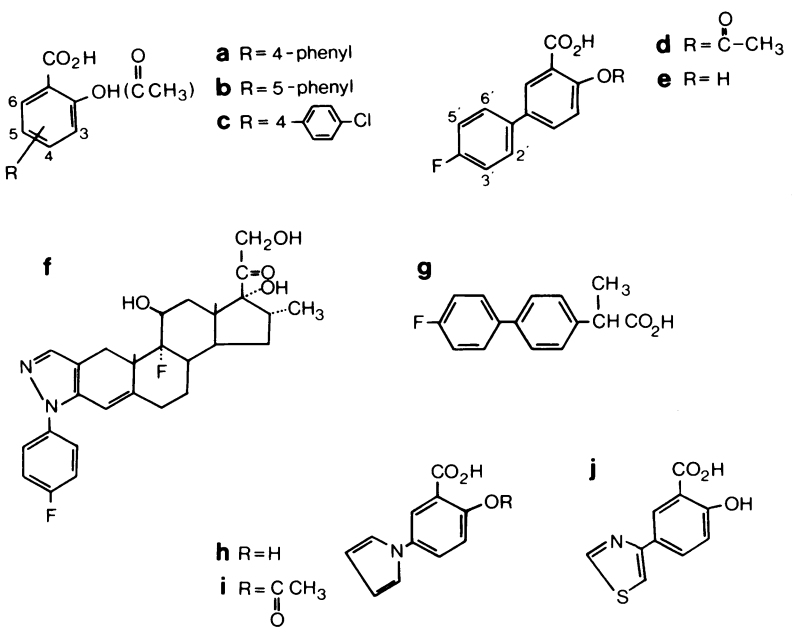


Figure 3 (a) 4-Phenyl salicylic acid; (b) 5-phenyl salicylic acid; (c) its 4-chloro derivative; (d) 5-*p*-fluorophenyl salicylic acid; (e) monofluoro analogue of diflunisal; (f) *p*-fluorophenyl (3,2-c)-pyrazole steroid; (g) 4′-fluorobiphenyl-α-propionic acid; (h) 5-(1-pyrryl) analogue of salicylic acid; (i) its *O*-acetyl derivative; (j) another heteroaryl analogue of salicylic acid.

additive. For example, combinations such as 3-methyl-5-phenyl and 5-benzyloxy-3-fluoro decrease, instead of increase, the activity of salicylates carrying a single substituent—fluoro, phenyl, methyl or benzyloxy only. Nevertheless, from the massive testing data obtained a tendency was found for hydrophobic substituents at the carbon-5 position to increase activity. Interestingly, similar preference of 5-alkyl (McCoubrey *et al.*, 1970) and 5-halo (Karler *et al.*, 1968) substituents was later observed in the inhibition of several enzyme systems by salicylic acid derivatives.

Synthesis of flufenisal

In the second phase of our chemical study special emphasis was given to the synthesis of the 5-*p*-fluorophenyl analogues (Figure 3d and e). The superiority of *p*-fluorophenyl over phenyl, possibly for electronic as well as metabolic reasons, has been demonstrated in many medicinal chemical studies. Its effectiveness in anti-inflammatory agents such as *p*-fluorophenyl(3,2-c)-pyrazole steroids (Figure 3f; Hirschmann *et al.*, 1963; Strachan *et al.*, 1964) and 4′-fluorobiphenyl-α-propionic acid (Figure 3g; Shen *et al.*, 1971) was also observed in our previous investigations. The synthesis of flufenisal was carried out in 1964 (Hannah *et al.*, 1970). Introduction of a *p*-fluorophenyl group at the carbon-5 position of aspirin resulted in a four-fold increase in potency in the carrageenin oedema and cotton pellet granuloma assays with an ED_{50} of 25 and 35 mg/kg, respectively. No significant difference in potency between the free phenol and its *O*-acetyl derivative was distinguishable in several animal assays, but it is notable that the free phenol was later found to be a more potent inhibitor of the prostaglandin (PG) synthetase *in vitro* (Ham *et al.*, 1972). Comparable activities were later shown by a group of related structures such as 2′-fluoro,2′4′-difluoro and 2′,3′,4′,5′,6′-pentafluoro derivatives of 5-phenylsalicylic acid (Ruyle *et al.*, 1973). Introduction of additional methyl or methoxy substituents to the structure in Figure 3d (flufenisal), decreased its activity only. The analgesia of flufenisal in the yeast-induced hyperaesthesia assay was estimated to be twice that of aspirin, and had a longer duration of action. The ED_{50} for flufenisal in the rat gastric haemorrhage assay was 200 mg/kg, which is almost 15 times less irritating than aspirin. In a preliminary clinical study, the increased analgesia and duration of action of flufenisal were readily confirmed in post-surgery patients (Bloomfield *et al.*, 1970). An attempt to establish its gastrointestinal tolerance in the chronic

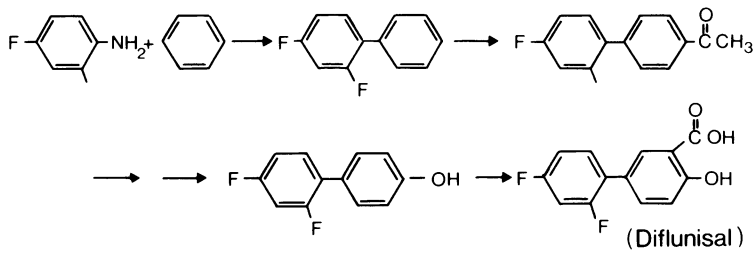


Figure 4 Synthetic procedure for diflunisal.

treatment of arthritis patients, however, was inconclusive. The development of flufenisal was further discouraged by its toxicity in chronic safety assessment. Both flufenisal and its *O*-acetyl derivative seemed to be as toxic as aspirin, at equipotent levels, in producing gastrointestinal lesions and papillary necrosis in rats and dogs.

5-Heteroaryl analogues and selection of diflunisal

After the encouraging, though disappointing, clinical experience with flufenisal, our search for a superior salicylate was resumed in 1969. A new synthetic chemical study focusing on the heteroaryl analogues was carried out (Walford *et al.*, 1971; Jones *et al.*, 1977). The replacement of a phenyl ring by a heteroaryl group has been widely used with success in the modification of many acidic as well as non-acidic anti-inflammatory agents (Scherrer & Whitehouse, 1974). Only a few heterocyclic analogues of substituted salicylates, among close to 100 synthesized, however, retained the desired properties (Table 1).

In analyzing the physicochemical parameters of these compounds (Table 1) the most important factors seemed to be a low basicity of the heteroaryl group, not protonated at the gastric acidity (*p*H 1–3), and an enhanced solubility in both aqueous and lipid media. In accordance with these general requirements was the promising activity of the 5-(1-pyrryl) analogue (Figure 3h) and its *O*-acetyl derivative (Figure 3i) in the acute anti-inflammatory and analgesic assays. Both compounds have ED_{50} in the carrageenin oedema assay at 40 mg/kg orally, almost twice as potent as aspirin. The analogue shown in Figure 3h is only one-tenth as potent as aspirin in the antipyretic assay but is 100 times less toxic in the gastric haemorrhage assay. A marked change in the pharmacological profile of aspirin by the 5-(1-pyrryl) substituent is clearly evident.

On re-examination of close analogues of flufenisal, the 2′,4′-difluoro derivative without an *O*-acetyl group (diflunisal) was found to be promising in several aspects. It is two to three times more potent than flufenisal, being active orally at 6–12 mg/kg in the carrageenin oedema, adjuvant arthritis and modified Randall–Selitto analgesic assays; and shows a low propensity to produce gastrointestinal irritations (Stone *et al.*, 1977).

A typical synthetic procedure of diflunisal is shown in Figure 4. Diflunisal is a stable white crystalline compound with a melting point of 211.5–212.5°C. Its ultraviolet absorption spectrum has λ_{max} at 228, 251 and 315 nm at A_{1167}, A_{564} and A_{130}, respectively. It is soluble in most organic solvents and dilute alkali. The aromatic fluorine atoms in diflunisal are chemically inert and relatively stable metabolically.

In diflunisal, the attachment of a 2,4-difluorophenyl group at the carbon-5 position of salicylic acid contributes much to its greater potency and longer duration of action. The omission of the *O*-acetyl group, commonly present in aspirin, benorylate, flufenisal (Figure 3d) and other active salicylates, was a deliberate choice primarily based on some safety considerations. The *O*-acetyl group in aspirin, since its introduction in 1889, has long been recognized as a chemical feature responsible for the improved

Table 1 Active heteroaryl salicylates

Compound	*Carrageenin oedema* ED_{50} *(mg/kg)*	*Gastric haemorrhage (mg/kg)*	*(incidence)*
Fig. 3h	40	1600	(0/5)
i	40	500	(4/5)
j	90	—	—
Aspirin	84	128	(4/5)

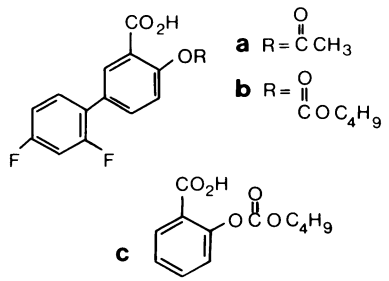


Figure 5 (a) *O*-acetyl derivative of diflunisal; (b) *n*-butyl carbonate derivative of salicylic acid; (c) *n*-butyl carbonate derivative of diflunisal.

tolerance and potency of aspirin over salicylic acid. We have found that, with 5-aryl or heteroaryl salicylates—for example, flufenisal and the pyrryl analogues, the activities of the free phenolic compounds (Figure 3e and h) and their *O*-acetyl derivatives (Figure 3d and i) are often comparable *in vivo*. Diflunisal seems to be slightly more active than its *O*-acetyl congener (Figure 5a) in animal models. Their ED_{50} in the oedema assay are 9 and 12 mg/kg, respectively. A choice between these two compounds, however, was not made simply on their minor difference in potency alone. Aspirin has a distinct advantage in pharmacokinetics over salicylic acid. The *O*-acetyl derivative (Figure 5a) might be expected to have a similar advantage over diflusinal. It also seemed that this analogue would be more likely to retain the pharmacokinetic characteristics of its monofluoro analogue, flufenisal, with a long duration of action. Until later clinical studies (Tocco *et al.*, 1975) the pharmacokinetics of the unacetylated diflunisal in man was less certain. On the other hand, a preference for diflunisal was strongly supported by some safety considerations.

Before our selection of diflunisal, the ability of aspirin to acetylate the macromolecules, serum albumin and platelet membrane, *in vitro* as well as *in vivo*, was reported by Farr and associates (Pinckard *et al.*, 1968; Hawkins *et al.*, 1969; Farr, 1971). The possible connection between this *in vivo* transacetylation and aspirin-induced toxicities—for example, asthma, anaemia, gastric haemorrhage, and platelet dysfunction—was cautioned (Farr, 1970; Krane, 1972). To avoid these potential side-effects in man, some of which are not readily detectable in short-term animal or clinical experiments, it seemed prudent to leave out the *O*-acetyl group from our new clinical candidate.

Another approach to retaining the pharmacokinetic characteristics of *O*-acyl salicylates but to minimize *in vivo* acetylation, was to use esters with very low potential for transacylation, such as the alkyl carbonates. Several carbonate esters of salicylic acid have been reported to be less reactive and less irritating derivatives (Dittert *et al.*, 1968). For example, the *n*-butyl carbonate (Figure 5c) is more resistant to chemical hydrolysis but is more readily cleaved by serum enzymes. In our study, the *n*-butyl carbonate of diflunisal (Figure 5b), with an ED_{50} of 14 mg/kg in the carrageenin oedema assay, is approximately equipotent with diflunisal on a molar basis, whereas the butyl carbonate of the compound in Figure 3h (5-(1-pyrryl) salicylic acid) is less active than that compound itself.

Both diflunisal and its pyrryl analogue (Figure 3h) were further compared in multiple biological assays and subacute safety assessment. On the basis of the overall efficacy and safety, diflunisal was finally chosen for clinical evaluation.

Further implications of the *O*-acetyl group

Our selection of non-acetylated diflunisal as the clinical candidate assumed a new meaning in the light of some recent biochemical experiments. The inhibition of PG biosynthesis by aspirin involves an irreversible acetylation of cyclo-oxygenase (Roth *et al.*, 1975). The platelet enzyme seems to be more sensitive to inactivation by aspirin than the same enzyme from sheep seminal vesicles (SSV) (Majerus & Stanford, 1977) and synovial tissues (Patrono *et al.*, 1976). In contrast, PG synthetase from these tissues are relatively sensitive to reversible inhibition by indomethacin and other arylacidic anti-inflammatory drugs. These observations accord with the more profound and longer lasting effects of low doses of aspirin on platelet functions in man. Unlike aspirin, diflunisal, without an *O*-acetyl group, is chemically incapable of acetylating cyclo-oxygenase, a fact clearly demonstrated in the next paper (Majerus & Stanford, 1977).

From the structure–activity relationship point of view, the effect of an *O*-acetyl group on the enzyme inhibitory action of diflunisal analogues *in vitro* is also opposite to that on aspirin. Salicylic acid is a very weak (1/100–1/10 times aspirin) inhibitor of cyclo-oxygenase. The addition of a phenyl substituent renders both diflunisal and the monofluoro analogue (Figure 3e) moderately potent reversible inhibitors (Ham *et al.*, 1972). Interestingly, both diflunisal and its monofluoro analogue are more potent than the acetylated flufenisal. Their inhibition of PG synthetase (from SSV) at 10 μg/ml is shown in Figure 6.

In comparing their chemical structures, it would seem that the binding site of the biphenyl carboxylic acids (diflunisal, its monofluoro analogue, and flufenisal) is not identical with that of aspirin. It also seems to differ partly in stereochemistry from that proposed for indomethacin and some phenyl-α-propionic acids (Gund & Shen, 1977). A similar

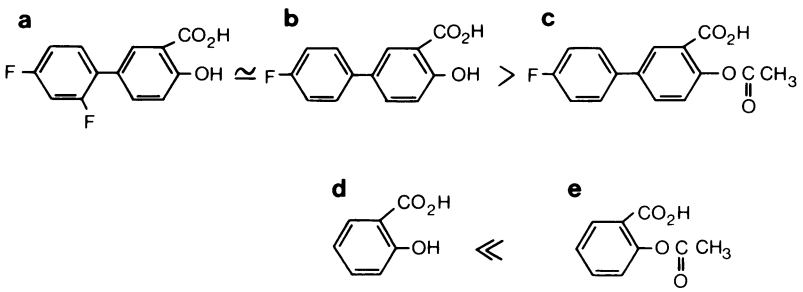


Figure 6 Inhibition of PG synthetase: (a) diflunisal (80%); (b) monofluoro analogue (75–80%); (c) acetylated flufenisal (48%); (d) salicylic acid (inactive); (e) aspirin (42%). Drug concentration: 10 μg/ml.

conclusion may be derived from the ineffective blockade of aspirin acetylation of cyclo-oxygenase by diflunisal (Majerus & Stanford, 1977). The lack of acetylation and the difference in binding by diflunisal compared with aspirin at the PG synthetase level may contribute to the better tolerance to diflunisal in man, particularly in terms of platelet dysfunction.

The inhibition of cyclo-oxygenase in PG synthesis is a plausible mechanism of action of aspirin-like drugs (Vane, 1971). The selective regulation of the subsequent conversions of the reactive hydroperoxide intermediates, PGG and PGH, to other PG metabolites provides other potential sites of action of anti-inflammatory agents. In addition to the PG pathway, the inhibition of leucocyte migration has been proposed as a possible mechanism of action of salicylates (Walker *et al.*, 1976). Further elucidation of the mechanism of action of diflunisal is in progress.

In conclusion, after an extensive medicinal chemical investigation of over 500 salicylate derivatives in current animal models, diflunisal, a novel non-acylating salicylate, has been found to be a promising anti-inflammatory-analgesic agent with an enhanced potency, and with longer duration of action and better tolerance than aspirin.

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