Chemistry and Biological Activities of N,N-Dimethylaminoethyl Acrylate, a Choline Acetyltransferase Inhibitor

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Received June 30, 1975

N,N-Dimethylaminoethyl acrylate (acryl-DMA) was synthesized as a tertiary nitrogen choline acetyltransferase (ChAc) inhibitor which would be able to penetrate biological membranes to inhibit ChAc in the nerve terminal. The synthesis from dimethylaminoethanol and acrylyl chloride was described and the hydration with times in an aqueous medium measured by NMR spectroscopy was presented. The autohydrolysis in water was found to be 1.75×10^{-8} mol/min at pH 7.4 and 5.0 m*M* concentration. The enzymatic hydrolysis was unaffected by cholinesterases. Acryl-DMA was capable of inhibiting ChAc extracted from rat brain with I_{50} of 5.02×10^{-4} *M*. The inhibition was reversible and displayed uncompetitive kinetics with respect to both substrates, choline and acetyl-CoA. Neither the hydrolysis nor the hydration products of acryl-DMA could inhibit ChAc. Although acryl-DMA was hydrated rapidly and completely within 1 hr at high pH (9.0), the time course of inhibition ability of acryl-DMA in aqueous medium at physiological pH was found to decrease rather slowly and by 36% in 1 hr, indicating that acryl-DMA can survive from hydration at physiological pH. Acryl-DMA was also tested for its ability to block electrically induced muscle contractions in both isolated skeletal and smooth nerve–muscle preparations. The ED_{50}'s obtained were less than 5×10^{-4} *M* in both cases.

Choline acetyltransferase (ChAc, acetyl-CoA:choline *O*-acetyltransferase, IUB 2.3.1.6) is the enzyme responsible for the synthesis of acetylcholine (ACh) from acetyl-CoA and choline. A number of compounds have been discovered which are able to inhibit ChAc and to decrease the synthesis of ACh in the cell-free preparation. However, since ChAc is located within the nerve terminal, any ChAc inhibitor which is capable of working in biological preparations must pass through the cell membrane to reach ChAc in the intracellular site. Therefore, a new ChAc inhibitor capable of penetrating cell membranes was sought.

Generally, there are two groups of ChAc inhibitors, the styrylpyridines[1] and the alkylaminoethyl esters.[2-4] Although styrylpyridines have been widely studied,[5-8] little attention has been paid to the alkylaminoethyl esters. Of the latter group, two quaternary amines have been reported to be potent and specific to inhibit ChAc. There are halogenoacetylcholines[3,10] and the naturally occurring ester, acryloylcholine.[4,9] Since these compounds are quaternary amines, they would not be expected to inhibit ChAc effectively in vivo.[10] The tertiary derivatives of these compounds, *N,N*-dimethylaminoethyl haloacetate and *N,N*-dimethylaminoethyl acrylate (acryl-DMA), respectively, would be expected to retain ChAc inhibitory activity while being able to penetrate the nerve terminal membrane to inhibit ChAc inside. The former compound has been studied briefly as a ChAc inhibitor in the past;[11] however, acryl-DMA has never been studied chemically or pharmacologically. Therefore, this report deals with the chemistry of acryl-DMA, its activity as a ChAc inhibitor, and its effect on biological preparations.

Results

Chemistry of Acryl-DMA. Acryl-DMA was prepared previously via a multistep, complex reaction in which β-chloropropionyl chloride was allowed to react with dimethylaminoethanol.[12] Attempts were made in our laboratory to find an easier method to synthesize acryl-DMA. Originally, preparation of the hydrochloride salt of the compound was attempted by adding acrylyl chloride to an equimolar amount of dimethylaminoethanol at 4°C in anhydrous diethyl ether. The white precipitate formed was extremely hygroscopic and turned yellow rapidly. The NMR analysis indicated an absence of vinyl protons.

Two alternate methods were then tried. In the first case, equimolar amounts of dimethylaminoethanol and acrylic acid were refluxed in dry benzene with *p*-toluenesulfonic acid to prevent its polymerization. The acryl-DMA was collected as the free base from vacuum distillation. In the second case, acryl-DMA was prepared by a condensation of dimethylaminoethanol and acrylyl chloride with triethylamine to trap the hydrogen chloride evolved during the reaction. The acryl-DMA so formed was separated by filtering off the triethylamine hydrochloride and distilled under vacuum to yield the free base. The product was verified by NMR spectroscopy and elemental analysis for C, H, and N ($C_7H_{13}NO_2$). The second method was better than the first one because it was easier and faster to perform. All of the acryl-DMA used in these studies was prepared by this method.

The stability of acryl-DMA was tested with NMR spectroscopy both as the free base and as a solution dissolved in chloroform-*d*, D_2O, and Me_2SO-d_6. Acryl-DMA remained unchanged for many days in all but the aqueous medium. To examine more closely the stability of acryl-DMA in water, NMR spectral analyses in D_2O were performed every 3 min for 1 hr. Since acryl-DMA is a base, the pH remained at 9.0 (±0.5) throughout the experiment. This indicates that the compound can survive from hydrolysis in aqueous medium during this time, because otherwise the pH should drop markedly. A series of the changes in spectra have occurred. There was a rapid emergence of a new singlet (at 2.9 ppm) between the methylene triplets (at 2.5 and 4.0 ppm), and this became more pronounced with time. Concurrent with this was the development of the new *N*-methyl proton singlet (at 2.2 ppm) slightly downfield from the original (at 2.0 ppm). After 1 hr, the new singlet had nearly replaced the old one and the vinyl proton region (at 6.0 ppm) had greatly decreased.

An analysis of this NMR study along with a consideration of the chemistry of acryl-DMA indicates that when acryl-DMA is dissolved in water, hydration occurs across the double bond of acryl-DMA to form *N,N*-dimethylaminoethyl β-hydroxypropionate. This reaction would occur via nucleophilic attack of a hydroxyl ion to the β carbon of the vinyl group. The reaction would be facilitated for two reasons. First, acryl-DMA is an α,β-unsaturated ester which can resonance stabilize the negative charge resulting from the nucleophilic attack. Second, since acryl-DMA, being a tertiary amine, is a fairly strong base, it can create an environment of the hydroxyl ion nucleophile by accepting a proton from the aqueous solution. The fact that acryl-DMA is stable in nonaqueous

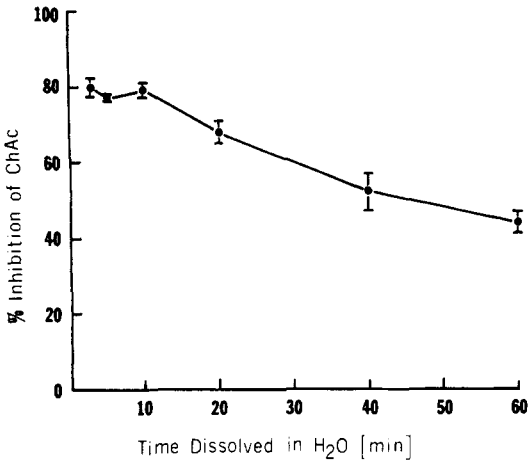

Figure 1. Decreases of ChAc inhibitory action of acryl-DMA in aqueous medium with time. Each point is a mean of three experiments and bars represent SEM. Acryl-DMA dissolved in H_2O was 4 m*M* and was incubated with ChAc during experiments at 1 m*M*.

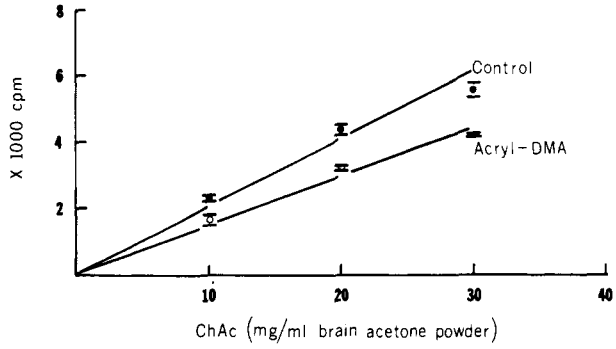

Figure 2. Reversibility of ChAc inhibition by acryl-DMA (5×10^{-4} *M*). Each point is a mean of three experiments and bars represent SEM.

medium supports the finding of the hydration reaction. In addition, acryloylcholine iodide, which is not a base, is much more stable than acryl-DMA in the aqueous medium.

Inhibition of ChAc by Acryl-DMA. The ability of acryl-DMA to inhibit ChAc was tested with the enzyme extracted from acetone powder of rat brain. The I_{50} for enzyme inhibition was 5.02 (±0.95) $\times 10^{-4}$ *M*. The hydrolysis products of acryl-DMA, dimethylaminoethanol and acrylic acid, were also tested for ChAc inhibitory activity and were found to cause less than 2% inhibition at 1.0 m*M* concentration. In addition, acryl-DMA was converted to the hydration product by reacting it with unbuffered water for 24 hr and then was tested as a ChAc inhibitor. Again, no inhibition was observed at 1.0 m*M* concentration. Therefore, it is apparent from these results that the inhibition of ChAc observed was due to the parent compound, acryl-DMA, but not due to the products of hydrolysis or hydration.

Table I. Rate of Hydrolysis of Acryl-DMA

Condition[a]	Hydrolysis rate,[b] mol/min × 10^8	
	Acryl-DMA	ACh
Autohydrolysis	1.75 ± 0.04	1.19 ± 0.03
AChE	0.07 ± 0.01	1.46 ± 0.03
BuChE	0.06 ± 0.01	6.57 ± 0.17

[a] Acryl-DMA and acetylcholine, 5×10^{-3} *M*; AChE, 0.083 U/ml; BuChE, 0.017 U/ml. [b] Mean ± SEM of three experiments.

To test the change in inhibitory activity with time at physiological pH, acryl-DMA was dissolved in water buffered at pH 7.4 for varying time periods (3, 5, 10, 20, 40, and 60 min) before adding it to the enzyme assay system. Figure 1 shows a gradual decrease in inhibitory activity with time under these conditions.

To further investigate the inhibition mechanism by this compound, the reversibility and kinetics were studied with the rat brain ChAc. Acryl-DMA was shown to be a reversible inhibitor as demonstrated with an Ackermann–Potter analyses[13] in Figure 2. Acryl-DMA is an uncompetitive ChAc inhibitor with respect to both substrates, acetyl-CoA and choline, as demonstrated with Lineweaver–Burk plots in Figure 3.

Hydrolysis of Acryl-DMA. In order to investigate the stability of the ester linkage, the rates of autohydrolysis and hydrolysis in the presence of acetylcholinesterase (AChE) and butrylcholinesterase (BuChE) were investigated. The results indicate that acryl-DMA is a poor

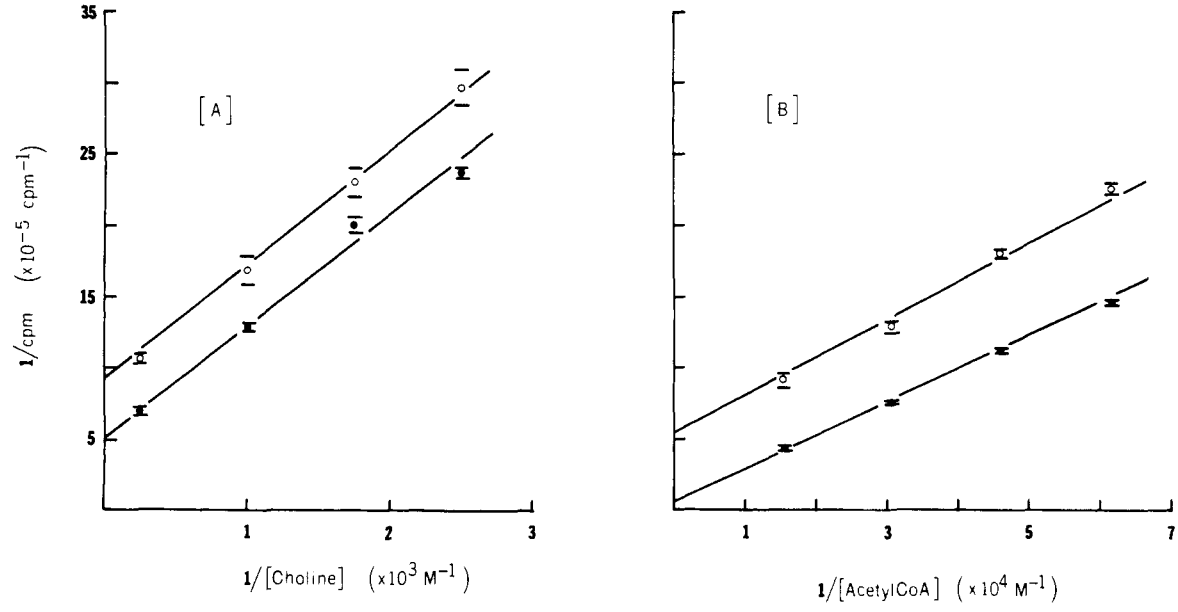

Figure 3. Kinetics of ChAc inhibition by acryl-DMA: (•) control; (○) acryl-DMA. [A] acryl-DMA, 0.2 m*M*; choline, 0.4–4.0 m*M*; acetyl-CoA, 0.65 m*M*. [B] acryl-DMA, 1.0 m*M*; choline, 25 m*M*; acetyl-CoA, 0.016–0.065 m*M*. Each point is a mean of three experiments and bars represent SEM.

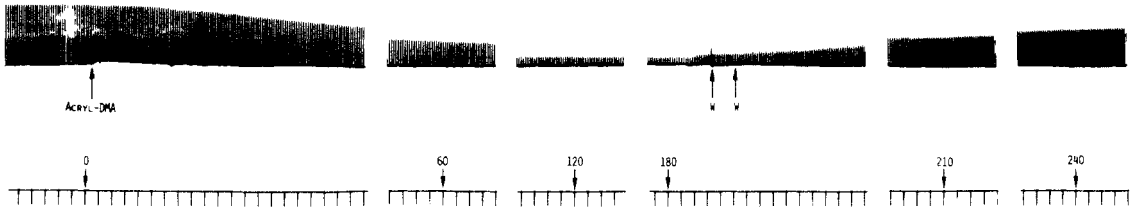

Figure 4. Effects of acryl-DMA on muscle contractions in the frog sciatic nerve–gastrocnemius muscle preparation in vitro: acryl-DMA, 1×10^{-3} M; W, washing. Each time interval represents 1 min.

Table II. ED_{50} of Acryl-DMA to Paralyze Cholinergic Neurons in Biological Preparations

Preparation	N	ED_{50},[a] $M \times 10^4$
Frog sciatic–gastrocnemius	16	5.05 (2.02-12.63)
Guinea pig ileum	20	1.21 (0.62-2.36)

[a] Means presented with 95% confidence limits in parentheses.[10]

substrate for the cholinesterases (Table I). The half-time of the autohydrolysis rate at pH 7.4 is 24 hr.

Effects of Acryl-DMA on Biological Preparations. Finally, acryl-DMA was investigated for its ability to block muscle contractions which were induced indirectly by electrical stimulation to the nerve. These studies were performed with both isolated skeletal muscle (frog sciatic nerve–gastrocnemius muscle preparation[14]) and smooth muscle (transmurally stimulated guinea pig ileum[15]). The ED_{50}'s for blockade in these two nerve–muscle preparations are presented in Table II. It was also found that acryl-DMA was reversible after wash-out (Figure 4), supporting the results of the enzyme inhibition study, and demonstrating that the blockade in the nerve–muscle system was a result of the continued presence of the inhibitor.

Discussion

Acryl-DMA was prepared as the tertiary nitrogen derivative of acryloylcholine for possible value as a ChAc inhibitor which could penetrate biological membranes to inhibit ChAc in the intracellular site. The pK_a of acryl-DMA (8.60) indicates that the compound is 94% ionized at physiological pH. The uncharged species would favor passage into the nerve terminal while the charged form would allow for ChAc inhibition.

The ester bond of acryl-DMA is relatively stable to hydrolysis. Rapid hydrolysis of AChE and BuChE is a major drawback to many of the choline ester analogs, but since acryl-DMA is unaffected by these enzymes, this would favor its use in vivo. The chemical changes of acryl-DMA which took place in aqueous medium at pH 9.0, as indicated by NMR spectroscopy, showed the emergence of a new compound, possibly the hydrated product of acryl-DMA, N,N-dimethylaminoethyl β-hydroxypropionate. These changes were not seen with acryloylcholine, indicating the sensitivity of nucleophilic addition at high pH, as would be expected.

When acryl-DMA was allowed to remain in aqueous medium at physiological pH for various time periods, the percent inhibition of ChAc decreased slowly by about 36% in 1 hr. Since neither products of hydration or hydrolysis inhibited ChAc, it indicates that the ChAc inhibition is induced by the parent compound, acryl-DMA, which is much more stable at physiological pH than in the high pH conditions. This is also indicated by the studies on the nerve–muscle preparation. More than 3 hr after addition of acryl-DMA to the organ bath, neuromuscular inhibition was still present; and since muscle contractions returned after the compound was washed out, the inhibition had to be due to the continued presence of the inhibitor.

Acryl-DMA was shown to be a reversible uncompetitive inhibitor of ChAc with respect to both acetyl-CoA and choline. All of the alkylaminoethyl esters investigated to date display uncompetitive kinetics,[3,4,11] suggesting that they all act via a common mechanism to inhibit ChAc.

Experimental Section

Materials and Equipment. All chemicals used in the synthesis and analysis of acryl-DMA are commercially available. pH determinations were made on a Beckman SS-3 pH meter with a Beckman combination electrode no. 39030. All NMR analyses were performed on a Varian A-60 spectrometer. The spectra were recorded at a sweep width of 500 Hz, sweep time of 250 sec, rf field of 0.05 mG, and a filter bandwidth of 4 Hz. Spectrum amplitudes were 1.6 for analysis of the pure free base of acryl-DMA and 10 for the time study of acryl-DMA in D_2O. Deuterated solvents were obtained from Bio-Rad Laboratories. AChE and BuChE were obtained from Nutritional Biochemical Corp. The radioisotopic [1-^{14}C-acetyl]-CoA was obtained from International Chemical and Nuclear Corp. and had a specific activity of 50 mCi/mmol. The isotope was counted with a Beckman Model 1650 liquid scintillation counter.

Synthesis. Dimethylaminoethanol (0.25 mol) was added to 300 ml of anhydrous diethyl ether at 0°C. An excess of triethylamine (0.30 mol) was added to the medium to trap the hydrogen chloride evolved in the reaction and to prevent formation of the HCl salt of acryl-DMA. Acrylyl chloride was added slowly with constant stirring to the medium such that the temperature never exceeded 5°C. After completion of the reaction, the triethylamine hydrochloride which formed as a white precipitate was filtered off and the ether was evaporated from the remaining solution under vacuum. The final product was obtained by distillation at 42°C at 1 mmHg with a 25% yield. Acryl-DMA is a clear colorless liquid with a mol wt of 143.21 and density of 0.925 g/ml with a bp of 161°C at 1 atm and a pK_a of 8.60. It is miscible in water, ethanol, acetone, Me_2SO, chloroform, and ether and can be stored for months at −80°C with no change.

Hydrolysis of Ester Linkage. The reaction was run at 25°C for 20 min with 10 ml of an aqueous barbital buffer solution (sodium barbitol 1.5 mM, KH_2PO_4 0.3 mM, NaCl 150 mM) containing either no enzyme, 0.83 units of AChE, or 0.17 units of BuChE. The pH was adjusted with 1 N HCl (0.05 ml) so that addition of the acryl-DMA gave a pH of 7.3–7.6. Acryl-DMA was added to give a final concentration of 5.0 mM and the change in pH with time was measured and compared with a standard curve obtained with identical solutions titrated with acrylic acid.

Activity of ChAc. ChAc was extracted from acetone powder of Holtzman rat brains[16] with ice-cold extraction medium (NaCl, 100 mM; EDTA, 1 mM; cysteine, 4 mM; bovine serum albumin, 0.05%; and NaH_2PO_4–Na_2HPO_4 buffer, 10 mM at pH 7.0) using 4 ml per 100 mg of acetone powder with constant stirring for 20 min according to a modified method of Burgen et al.[17] The extract was centrifuged at 9000 g at 4°C for 10 min and the supernatant fraction containing ChAc was retained. Acetylcholine synthesis was determined by radioassay using a modified method of Schrier and Shuster.[18] The enzyme extract (0.05 ml) was preincubated with the inhibitor (or water in the case of control) in 0.2 ml of incubation medium (NaCl, 150 mM; neostigmine, 0.05 mM; choline, 50 mM; and sodium phosphate buffer pH 7.0, 25 mM). After 20 min of preincubation at 37°C, 0.01 ml of [1-^{14}C-acetyl]-CoA was added to give a final concentration of 0.065 mM containing 0.15 μCi radioactivity. The reaction was continued at 37°C for 30 more min and was then stopped by adding 4.3 ml of ice-cold water and, immediately thereafter, 500 mg of Bio-Rad

AG 1 × 1 anion exchange resin to remove unreacted acetyl-CoA. After vigorous stirring for 30 sec, each incubation tube was centrifuged for 5 min at 1500 g. Then, 0.5 ml of the supernatant fraction was transferred to 10 ml of scintillation solution (2,5-diphenyloxazole, 7 g; naphthalene, 100 g; dioxane, 1000 ml) and counted in a liquid scintillation counter. The remaining supernatant was transferred to a clean centrifuge tube and 250 mg of Amberlite CG 120 cationic exchange resin was added to trap choline derivatives. After stirring and centrifuging as above, 0.5 ml of the supernatant was again added to 10 ml of scintillation solution for counting. The amount of acetylated product was calculated by subtracting the cpm obtained after both anionic and cationic exchange from the cpm obtained after anionic exchange alone. (10000 cpm is equivalent to 1.04 nmol of acetylated product synthesized/min/mg of acetone powder).

For studies on the reversibility of acryl-DMA, the concentration of ChAc varied from 10 to 30 mg of acetone powder per milliliter. For studies on the inhibition kinetics of acryl-DMA, either the acetyl-CoA concentration varied from 0.016 to 0.065 m*M* while the concentration of choline remained constant at 25 m*M*; or the choline concentration varied from 0.4 to 4.0 m*M* while the acetyl-CoA concentration remained constant at 0.65 m*M*.

Frog Sciatic Nerve–Gastrocnemius Muscle. *Rana pipiens* weighing 20–25 g were stunned, decapitated, and pithed. The muscle with sciatic nerve was removed and suspended in an organ bath in 40 ml of frog Ringer solution at 25°C oxygenated with 95% O_2–5% CO_2. The sciatic nerve was stimulated with a pair of platinum ring electrodes with 7-V voltage, 0.5-msec duration, and 250-Hz frequency administered for 0.1 sec every 10 sec. Muscle contractions were measured with a Narco Bio-Systems myograph-B isometric force transducer and recorded on a Model Four-A Narco Bio-Systems physiograph. Acryl-DMA was added to the bath in volumes of not exceeding 0.8 ml.

Transmurally Stimulated Guinea Pig Ileum. Guinea pigs (common strain), 300–500 g, were stunned with a blow to the head and decapitated. The abdominal cavity was opened to expose the intestine. A terminal portion of the ileum was cut into 20–30 mm sections, tied on either end with silk suture, and suspended in an organ bath of 40-ml capacity. The ileum was mounted on a coaxial electrode such that transmural stimulation could be administered to the nerves. The ileum was bathed with Tyrode solution oxygenated with 95% O_2–5% CO_2 at 37°C. The nerves were stimulated with monophasic square wave pulses administered at a supramaximal voltage of 40 V, frequency of 10 Hz, and 0.5-msec duration for 1 sec every 10 sec. Muscle contractions were recorded as with the frog muscle preparation, and acryl-DMA was added to the bath in volumes not exceeding 0.8 ml.

Statistical Analyses. Standard errors of the mean (SEM) were given for mean figures. Confidence limits at the 95% level were determined for the ED_{50} values according to Litchfield and Wilcoxon.[19]

Acknowledgment. The authors are grateful to Mr. Charles E. Strattan for his excellent technical assistance. Mr. P. P. Rowell was supported by a NIH predoctoral training grant (GM-00760). This work was supported in part by research grants from National Institutes of Health (CA-17584 and NS-09302).

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Antiinflammatory Activity of Some 2,3-Dihydrobenzofuran-5-acetic Acids and Related Compounds

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A series of 2,3-dihydrobenzofuran-5-acetic acids and related compounds was prepared as potential antiinflammatory agents. As measured by the carrageenan-induced edema method for the preliminary screening test, introduction of a methyl group α to the acetic acid function enhanced the antiinflammatory activity, and α-(7-chloro-2,2-dimethyl-2,3-dihydrobenzofuran)-α-methyl-5-acetic acid (**13a**) showed the most potent activity in this series.

Among the reports of compounds exhibiting antiinflammatory activity which have appeared in recent years, a number describe compounds which belong to the aryl- or heteroarylalkanoic acids.[1] Features of typical molecules which are important for the activity include a carboxyl group separated by one or more carbon atoms from the aromatic nucleus, which is further substituted by a relatively large lipophilic group at its meta or para position.

This paper describes the synthesis and antiinflammatory activity of 2,3,7-substituted 2,3-dihydrobenzofuran-5-acetic acids and related compounds (type 1). First of all, in order to investigate the effects of introducing a methyl group at position 2 or 3 in the 2,3-dihydrobenzofuran ring on the activity, 2-methyl-, 2,2-dimethyl-, and 2,3-dimethyl analogs were prepared.

Introduction of a halogen atom at the position meta to the acetic acid residue tends to increase the activity as is known in alclofenac[2] and flurbiprofen;[3] consequently, 7-chloro analogs were synthesized to investigate the effect of chlorine at position 7 in 2,3-dihydrobenzofurans. It is