Registry No. 1a, 109363-32-8; 1b, 85835-41-2; 2a, 109334-88-5; 2b, 107941-76-4; 3a, 109334-89-6; 3b, 92535-15-4; 4a, 109334-90-9; 4b, 109335-03-7; 5, 148-82-3; 6, 109334-91-0; 7, 109334-92-1; 8a, 109334-93-2; 8b, 28635-78-1; 9, 107941-82-2; 10, 109334-94-3; 11a, 88282-65-9; 11b, 109363-33-9; 12a, 109334-95-4; 12b, 109335-04-8; 13, 6671-25-6; 14, 109334-96-5; 15, 109334-97-6; 16, 109334-98-7; 17a, 109334-99-8; 17b, 109363-34-0; 18a, 109335-00-4; 18b, 109335-05-9; 19, 109335-01-5; 20a, 109335-02-6; 20b, 109335-06-0; CbzCl, 501-53-1; H-Leu-OBu-*t*·HCl, 2748-02-9; $PhCH_2Br$, 100-39-0; Cbz-Tyr(Bu-*t*)-OH, 5545-54-0; $H_2C{=}CHCH_2Br$, 106-95-6.

Notes

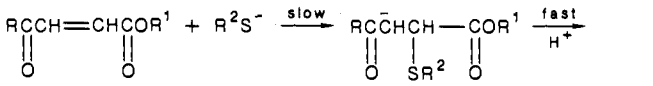
3-Carbonylacrylic Derivatives as Potential Antimicrobial Agents. Correlations between Activity and Reactivity toward Cysteine

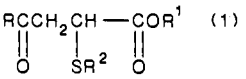
Alma Dal Pozzo,* Maurizio Acquasaliente, Giovanni Donzelli, Paolo DeMaria,† and M. Cristina Nicoli

Istituto di Chimica e Biochimica G. Ronzoni, 20133 Milano, Italy, and Istituto di Scienze Chimiche, Università di Bologna, 40127 Bologna, Italy. *Received December 3, 1986*

A number of 3-carbonylacrylic acid derivatives were prepared, with a view to varying systematically the stereoelectronic environment of the conjugated double bond. The rates of reaction with cysteine were measured spectrophotometrically when possible or by stopped flow when very fast. Some of the final reaction products were isolated. Other properties examined were partition substituent constants and antimicrobial activity. On the basis of published data and these studies, the activity appears to be the combined effect of at least two mechanisms, one probably related to the effect of these structures on surface tension, the other to the electrophilic properties of the unsaturated system.

The potential antimicrobial activity of α,β-unsaturated carbonyl compounds continues to receive attention, and several substances containing this function, obtained either from natural or synthetic sources, are currently used in therapy.[1] It is generally assumed and confirmed by experimental evidence that the activity of this class of compounds is due to alkylation of nucleophilic groups, such as amino groups, or, preferably, sulfhydryls of essential enzymes.[2-8] The reaction involves a Michael-type addition of the nucleophile to the activated double bond of 3-carbonylacrylates, according to the scheme





(1)

It should be noted that Michael-type additions are often reversible, meaning that these substances can yield reversible macromolecular complexes and, hence, cannot be strictly considered irreversible alkylating agents.[2,9] On the other hand, the studies of Lee et al.[10,11] established that these unsaturated structures exert their biological effect by inhibiting enzyme activities, which control cell division without alkylating or impairing DNA template function. These two aspects make compounds containing the α,β-unsaturated carbonyl moiety attractive among alkylating agents. Unfortunately, they share with the latter the lack of selectivity, certainly because their high degree of reactivity leads to indiscriminate reactions with many cell constituents. In this respect, the extensive synthetic efforts to date have led to only minimal improvements over the prototype drugs.

Several systematic studies on unsaturated carbonyl derivatives failed to establish definite reactivity–activity relationships.[12-14] This lack of correlation may mean that differences in reactivity toward nucleophiles, in themselves, do not necessarily have a determining influence upon biological activity, when considered independently from other structural parameters. Furthermore, any substitution in these highly reactive molecules creates a marked structural diversity, precluding the determination of most of the free-energy parameters required for a rigorous Hansch analysis.[15]

As far as aroylacrylates are concerned, few studies have assessed activity–structure relationships. In 1949 Kirchner and Cavallito[2] found a good correlation between antimicrobial activity and chain length of alkyl substituents in the para position of 3-benzoylacrylic acid, and in 1979 Bowden et al.[16] correlated activities and partition sub-

†Present address: Istituto di Chimica Organica, Università di Palermo, 90123 Palermo, Italy.

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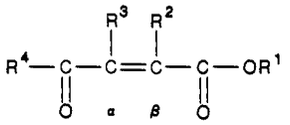
Table I.[a] Reaction Rates with Cysteine, Partition Coefficients, and Bacteriostatic and Fungistatic Activities of (*E*)- and (*Z*)-3-Carbonylacrylic Acids and Their Methyl Esters



no.	configuration	R^4	R^3	R^2	R^1	physicochemical param: k_2, L mol^{-1} s^{-1}	physicochemical param: log P[b]	MIC, μmol/mL: *S. aureus* ATCC 6538	MIC, μmol/mL: *S. pyogenes* A[g] IVM P 134	MIC, μmol/mL: *M. canis* ATCC 11621	expt condtns for k_2 measurements: meth	temp, °C	λ, nm
1	*Z*	$CH_3(CH_2)_3$	CH_3	CH_3	H	ndr[c]	0.9		3.8[f]		III[d]	22	343
2	*Z*	$CH_3(CH_2)_6$	CH_3	CH_3	H	ndr	2.4		0.32[f]		III[d]	22	343
3	*Z*	$CH_3(CH_2)_{10}$	CH_3	CH_3	H	ndr	4.4		0.01[f]		III[d]	22	343
4	*Z*	C_6H_5	CH_3	H	H	ndr	1.69	>16	2.2	16	I	60	265
5	*Z*	4′-$CH_3C_6H_4$	CH_3	H	H	ndr	2.19	>15	2	3.2	I	60	275
6	*E*	C_6H_5	CH_3	H	H	0.001[e]	2.44	18	2		I	60	285
7	*E*	4′-$CH_3C_6H_4$	CH_3	H	H	0.001[e]	2.94	15		3.2	I	60	275
8	*Z*	4′-$CH_3C_6H_4$	H	CH_3	H	0.005[e]	2.19	15	2	3.2	I	22	285
9	*E*	4′-$CH_3C_6H_4$	H	CH_3	H	0.035	2.94	16	2	1.2	I	22	285
10	*Z*	C_6H_5	H	H	CH_3	3.17	1.49	0.04	0.003	<0.002	II	25	265
11	*E*	4′-$CH_3C_6H_4$	H	H	H	85.6	2.44	1	0.013	1	II	25	290
12	*E*	4′-$C_6H_5C_6H_4$	H	H	H	85.9	3.9	0.08	0.006		II	25	320
13	*E*	C_6H_5	H	H	H	144	1.94	1.26	0.03	1	II	25	290
14	*E*	3′-C_5H_4N	H	H	H	282	0.26	1	0.05		II	25	280
15	*E*	4′-$CH_3C_6H_4$	H	H	CH_3	908	2.74	0.05	0.024	0.05	II	25	295
16	*E*	C_6H_5	H	H	CH_3	1560	2.24	0.04	0.010	0.0026	II	25	290
17	*E*	3′-C_5H_4N	H	H	CH_3	2620	0.56	0.16	0.042	0.005	II	25	290

[a] Positions α and β were considered related to the carbonyl group in position 3. [b] Log *P* values were partly measured experimentally, partly calculated according to Leo et al. [c] No detectable reaction. [d] These compounds were dissolved in buffer containing 20% dioxane. [e] The reaction reaches the equilibrium. [f] Ref 21. [g] *Streptococcus pyogenes A.*

stituent constants of some aroylacrylic acids by a Hansch-type linear free energy equation. However, as far as reactivity is concerned, the members of both series considered do not have significant differences. In order to elucidate the reactivity–activity relationship of this class of compounds, it seemed desirable to prepare a number of substituted as well as unsubstituted 3-carbonylacrylates differing significantly in terms of the electronic and steric environment of the double bond and to study which factors regulate the rate of the nucleophilic addition. Particular attention was placed on the stereoelectronic effects of the methyl substitution in the α- or β-position, connected with configurational effect. We have therefore undertaken kinetic measurements of the reaction with cysteine and compared the results with the log *P* and the antimicrobial activity.

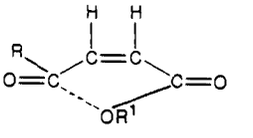
Results and Discussion

All the data related to the carbonylacrylic acids and their esters, namely, the second-order rate constants of the reaction with cysteine, the log *P* values, and the antimicrobial activity against three representative microorganisms, are reported in Table I. The current data on the activity of compounds **4–13**, **15** and **16** against the growth of *Staphylococcus aureus* and *Microsporum canis* are largely in agreement with those previously reported by other authors.[17] The compounds were listed in Table I in order of their increasing k_2, which we assumed to be the most significant parameter in relation to the antimicrobial activity.

Correlations of Structure and Reactivity. The results of the kinetic measurements in most cases confirmed the expectations, but some compounds gave unexpected and interesting results. The correlations between structure and reactivity toward the nucleophile can be discussed with consideration of the following points.

1. Effect of substitution on the double bond: The reaction with cysteine of all carbonylacrylic derivatives containing the unsubstituted double bond is fast and goes to completion, whereas it decreases dramatically when one hydrogen atom of the double bond is replaced by a methyl group. For example, by comparing compounds **9** and **11**, it can be noticed that k_2 becomes 2500 times lower when position 2 (β) is occupied, due probably to steric factors. When position 3 (α) is occupied, as in compounds **4–6** and **7**, the reaction becomes even slower and it eventually reaches an equilibrium at about 20% of substrate consumption. The α-substitution induces more marked effects, because the transition state of the reaction is carbanionic; the electronic effect at the carbanion is clearly more important than the steric effect in the β-position. When both α- and β-positions are occupied by methyl groups, as in compounds **1–3**, the reaction is too slow to be measured.

2. Effect of configuration: It was unexpected that the *Z* isomer afforded in every case a much slower reaction than the corresponding *E* isomer, i.e., 7 times slower for compound **8** and 500 times slower in the case of compound **10**. (Reactions of compounds **4** and **5** were too slow to be detected.) Increased delocalization of the three conjugated double bonds and proximity effects could provide a higher stabilization to the ground state in the *Z* isomer such as shown below:



The possibility of a ring conformation in the transition state of 3-benzoylacrylic esters leading to a degree of electron delocalization has been previously demonstrated.[18]

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An alternative interpretation is that steric hindrance in the *Z* isomer may disturb the coplanarity of the α,β-unsaturated carbonyl, thus decreasing the electrophilicity at the β-position.

3. Effect of the replacement of the carboxyl with the ester group: The methyl esters **15–17** were far more reactive than their corresponding acids. Under the experimental conditions, all the carboxylic acids were present in the fully dissociated form. Hence, electrostatic repulsion between the conjugated base of the substrate and the negative charge present on the sulfur atom of the nucleophile may be responsible for the lower reactivity of the acids compared to the esters.

4. Effect of the substitution on the carbonyl group in position 3. Electron-donating substituents in the aromatic ring, as in compounds **11** and **12**, lower the reaction rates of the acids as well as the esters. In fact, the k_2 values of compounds **11** and **12** are approximately half that of compound **13**, and the ratio between the rate constants of compounds **15** and **16** is the same. On the other hand, electron-withdrawing groups increase the reactivity about two-fold in the cases considered (compounds **14** and **17** compared to **13** and **16**, respectively).

Correlation of Reactivity with Antimicrobial Activity. A certain degree of positive correlation was noted between the chemical reactivity with cysteine of the compounds listed in Table I and their antimicrobial activity. However, no obvious relationship exists among all members of the synthetic series: compounds with very little or no reactivity toward cysteine also exhibited a good level of activity, as in the case of compounds **1–9** As a consequence, it is reasonable to suppose that their antimicrobial activity depends on a mechanism of action other than nucleophilic addition on key enzymes. Compounds **1–9** are molecules bearing polar groups with hydrophobic substituents (R^4), and this structure suggests that they might be able to damage microbial membranes, like anionic detergents. If this were the case, the presence of the conjugated double bond could be of secondary or no importance in contributing to the antimicrobial activity. The ability of anionic detergents to penetrate the hydrophobic core of the membrane and to destroy the permeability barrier appears to depend on the length of the side chain. If one considers that compounds **1–3** belong to a homologue series, one can notice a clear dependence of the activity on log *P*, because the activity increases with the length of the aliphatic chain. Moreover, the latter compounds closely resemble the lichesterinic acids, whose unsaturated structure has been shown to be less important than their surface active properties in contributing to antibacterial activity.[19]

On the other hand, compounds **10–17** gave fast reactions with cysteine and their antimicrobial action has been previously demonstrated to be dependent on nucleophilic addition.[6,7]

It appears from Table I that, when log *P* values do not differ significantly, the activity increases with the reactivity. However, in the cases where lipophilicity is very high (compound **12**) or very low (compounds **14** and **17**), it apparently affects the activity. The most interesting behavior observed among all compounds considered is that of the *Z* isomers. As reported above, these isomers have the most unfavorable nucleophilic addition; nevertheless, they exhibit antimicrobial properties comparable to those of the *E* isomers, except for compound **10**, which was shown to have an even better activity. A possible explanation for these results is that the *Z* configuration gives rise to highly favorable interactions. This configurational effect is also present in compounds **4**, **5**, and **8**, whose activity has been mainly attributed to a membrane effect; therefore, the favorable interactions referred to before might be exerted at membrane level. This observation may be crucial in view of preparing new compounds with improved selectivity.

In conclusion, the systematic modification of the parent compound 3-carbonylacrylic acid and the study of the reactivity of all derivatives with cysteine, compared with antimicrobial activity and log *P*, can provide a basis to the understanding of the mechanism of action of these interesting antimicrobial agents and to any further biological study. The results support the hypothesis that all compounds belonging to this class possess at least two different mechanisms of action. The first, sensitive to chain length and steric effects, is a general surfactant action; the second, when present, consists in alkylation of nucleophilic groups of biologically essential macromolecules. When both mechanisms are operating, a cooperative effect takes place, so that the first mechanism facilitates the second, leading to substantial levels of antimicrobial activity.

Experimental Section

Materials. Compounds **1–3**, **14**, and **17** have been previously reported by our laboratory.[20,21] Compounds **4–13**, **15**, and **16** have been prepared according to published procedure.[18,22,23] The physical constants of all compounds agreed well with literature values. DL-Cysteine hydrochloride hydrate and Aldrithiol were supplied by Aldrich. Dioxane was purified according to the method of Vogel (*Pract. Org. Chem.*; Longmans: London).

Kinetic Measurements. In all cases the solvent used was an aqueous 0.06 M phosphate buffer at pH 7.4 with the addition of 2% (v/v) dioxane, except for compounds **1–3**, for which 20% dioxane was used, because of their very low solubility; in this case, the solvent effect was not detectable, because the compounds did not react with cysteine. However, for compounds soluble in both solvent systems (i.e., **13**) we have evidence that there is an approximately 30% increase in rate on passing from 2% to 20% dioxane. The kinetic measurements were carried out under pseudo-first-order conditions. The compounds are stereochemically stable under the conditions of the experiments over the reaction period.

Spectrophotometric Method (I). When possible, the reactions were followed spectrophotometrically by using a Unicam SP 8005 spectrophotometer, and the cuvette temperature at 22 °C was controlled by means of a thermocirculator; when the reaction was very slow, kinetics were determined at 60 °C. The solutions of cysteine, at concentrations varying from 3×10^{-4} to 3×10^{-2} M, were pipetted into 1-cm cuvettes, which were closed with silicon rubber serum caps. The solutions were purged by passing "oxygen-free nitrogen" for 1 min, with hypodermic needles being used for inlet and exhaust. The substrate (10 μL) under investigation was added by using a Hamilton microsyringe, so that the final substrate concentration was approximately 3×10^{-5} M. The UV spectrum in the range of 200 to 450 nm was recorded continuously. This enabled substrate and product spectra to be recorded. Rate measurements were made by using the procedure described above, but with monitoring at selected wavelengths based on the maximum exchange in absorption between the substrate and the product. Optical density was displayed on a Unicam AR recorder as a function of time, and the final optical

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Table II

adducts of compound no.	formula	anal.	mp, °C	UV λ_{max}, nm	TLC:[a] R_f	^{1}H NMR (Me_2SO-d_6, 5%, ppm)
10	$C_{14}H_{17}NO_5S$·HCl	CHNS	138–140	242	0.42	8.1–7.3 (m, Ar H), 4.1 (t, CHS), 3.85 (t, C*H*NH_2), 3.75–3.35 (m, OCH_3 + $COCH_2$), 3.15 (d, CH_2S)
16	$C_{14}H_{17}NO_5S$·HCl	CHNS	138–140	242	0.42	8.1–7.3 (m, Ar H), 4.1 (t, CHS), 3.85 (t, C*H*NH_2), 3.75–3.35 (m, OCH_3 + $COCH_2$), 3.15 (d, CH_2S)
17	$C_{13}H_{16}N_2O_5S$·HCl	CHNS	163–165	265	0.21	9.1, 8.7, 8.2, 7.3 (m, heterocyclic H), 4.1 (t, CHS), 3.85 (t, C*H*NH_2), 3.75–3.35 (OCH_3 + $COCH_2$), 3.15 (d, CH_2S)

[a] Silica gel 60 F_{254} Merck plates; mobile phase, benzene/methanol/acetic acid, 6:3:1; visualization, UV_{254} light; ninhydrin solution.

density was normally assumed to be that measured after "10 half-lives" had elapsed. In certain cases, the reaction reached an equilibrium; in these cases, the rate of the inverse reaction k_{-1} was calculated by plotting the rate constant k_{obsd} against the molar concentration of cysteine and evaluating k_{-1} at the intercept. In this way, the correct value of k_1 was obtained from the formula $k_1 = k_{obsd} - k_{-1}$, and the second-order rate constant k_2 was obtained from the slope of the linear plots of k_{obsd} vs. cysteine concentration. The wavelength employed and temperatures are reported in Table I, with the rate coefficient calculated.

Stopped-Flow Method (II). Some of the reactions were too fast to be measured by the spectrophotometric method described above. These were measured with a JASCO stopped-flow apparatus, calibrated by measuring known rates of reduction of 2,6-dichlorophenol/indophenol by L-ascorbic acid in several buffered aqueous solutions (rate constants were in agreement with literature values).[24] The stopped-flow measurements were made with the separated solutions of cysteine and substrate dissolved in buffered aqueous solutions with 2% dioxane, in concentrations double the final one and placed in the two reservoirs of the apparatus, so that the final concentration after mixing could be calculated. The experiments were conducted in pseudo-first-order conditions, with a large excess of cysteine (at least tenfold). The cysteine solutions were saturated with argon before use to avoid oxidation. The decrease in substrate concentration vs. time for at least six different concentrations of cysteine was read on a Tectronix Model 468 digital storage oscilloscope. The second-order rate constants k_2 were obtained as the slopes of linear plots of k_{obsd} vs. the molar concentrations of cysteine.

Aldrithiol Method (III). This method was used when there were no suitable observable changes in UV spectra. In this case, the consumption of cysteine was followed through its reaction with 2,2′-dipyridyl disulfide (Aldrithiol), with measurement of the resulting extinction at 343 nm.[13,25] A good agreement between this method and the spectrophotometric one was obtained in measuring the reaction between cysteine and (*E*)-3-(4-methoxybenzoyl)acrylic acid (unpublished results). The starting concentration of cysteine was always 10^{-4} M, and the concentrations of acrylic derivatives varied from 0.5 to 2×10^{-2} M. Both reagent solutions were prepared in a concentration double the final one, and they were mixed together in equal volume in a flask kept in a thermostat at 22 °C under argon. The decreasing concentration of cysteine was followed by pipetting at intervals 3 mL of the solution from the flask containing the reaction solution into the cuvette, and immediately 10 μL of a solution of Aldrithiol in dioxane was introduced, so that the final concentration was 5×10^{-4} M. The introduction of the final reagent stops the reaction and irreversibly forms 2-thiopyridone, whose absorption was measured by recording its spectrum in the range 400–300 nm.

Isolation of Products. Four of the final products of the reaction of the carbonylacrylic derivatives with cysteine (see eq 1) were isolated as white powders. Each UV spectrum perfectly overlapped to the spectrum recorded as a final product of the reaction between the two corresponding starting reactants by the Spectrophotometric Method described above. The NMR spectra showed the disappearance of doublets from CH=CH protons in all of the four adducts. The two isomer compounds (*Z*)- and (*E*)-3-benzoylacrylic ester produced apparently the same addition product. In fact, their NMR spectra were perfectly overlapping, like their UV spectra, and they showed the same R_f on TLC. Nevertheless, it is not possible to exclude, on this basis, differences in the configuration for the two adducts, and we are committed to investigate this point with further experimental work.

Adduct of (*E*)-3-Benzoylacrylic Acid (13). Compound **13** was obtained according to the method of Cavallito and Haskell[26] and crystallized from 75% ethanol: mp 168–172 °C; ^{1}H NMR (Me_2SO-d_6, 5%) 8.1–7.3 (aromatic protons), 6.2 (broad peak from mobile H's), 3–4 (complex pattern of signals from two CH_2CH groups) ppm.

Adducts of Methyl (*E*)-3-Nicotinoylacrylate (17) and Methyl (*Z*)- and (*E*)-3-Benzoylacrylate (10 and 16). General Procedures. Cysteine hydrochloride hydrate (92.44 mg, 0.525 mmol) was added to a solution of the unsaturated esters (0.525 mmol) in 2 mL of THF/H_2O (80:20). The reaction mixture was kept at room temperature, in subdued light, with stirring under argon, for 1 h, and then evaporated at reduced pressure. The residues contained the adducts in a pure state. Physicochemical constants are reported in Table II.

Biological Activities. As a measure of antimicrobial activity, the minimum inhibitory concentration (MIC), determined by the serial dilution technique in liquid medium, was used. All the MIC data are reported in Table I, expressed as micromoles/milliliter.

Partition Coefficient Values. The partition coefficients in octanol/water of (*E*)-3-benzoyl- and (*E*)-3-nicotinoylacrylic acids (**13** and **14**, respectively) were determined experimentally, according to the standard procedure described in the literature,[15] by using the equation

$$P = C_{oct}/C_{H_2O}(1 - \alpha)$$

where α is the dissociation degree of the acid, based on the pK_a of the compound. The pK_a's were determined potentiometrically, at 22 °C. Aqueous solutions of the two compounds containing 2% EtOH were titrated by KOH standard solution. The pK_a's found were 3.50 ± 0.03 and 3.82 ± 0.03 for compounds **13** and **14**, respectively.

Acknowledgment. This work was carried out with Italian CNR aid, special project on Fine Chemicals, and with M.P.I. financial support.

Registry No. 1, 109151-37-3; **2**, 109151-38-4; **3**, 109151-39-5; 4, 35509-85-4; **5**, 35513-29-2; **6**, 35504-92-8; **7**, 35504-95-1; 8, 35509-83-2; **9**, 19405-15-3; **10**, 19522-28-2; **10** (cysteine hydrochloride adduct), 109151-42-0; 11, 20972-36-5; **12**, 71149-96-7; **13**, 17812-07-6; **14**, 109151-40-8; **15**, 32149-28-3; **16**, 19522-25-9; **17**, 109151-41-9; **17** (cysteine hydrochloride adduct), 109151-43-1; cysteine, 52-90-4; cysteine hydrochloride, 52-89-1.

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