Table I

Compd	Mp, °C	% yield	Formula[d]
3 (n = 0)	152–154[a]	11	$C_{22}H_{27}NO_4$[e]
3 (n = 2)	144–146[b]	14	$C_{24}H_{31}NO_4$
4 (n = 0)	215–216[a]	25	$C_{27}H_{29}NO_4$
4 (n = 1)	213–217[c]	64	$C_{28}H_{31}NO_4 \cdot HCl$
4 (n = 2)	240–243[c]	57	$C_{29}H_{33}NO_4 \cdot HCl$
4 (n = 3)	200–205[c]	51	$C_{30}H_{35}NO_4 \cdot HCl \cdot H_2O$
4 (n = 4)	230–234[c]	53	$C_{31}H_{39}NO_4 \cdot HCl \cdot H_2O$

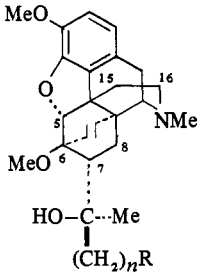
Recrystallization solvent: [a]EtOH; [b]MeOH; [c]EtOH–Et_2O. [d]Analyses for C, H, N. [e]C: calcd, 71.52; found, 71.04.

Table II

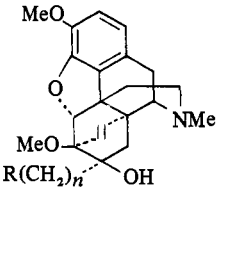
Structure	Analgetic[a] ED_{50}, mg/kg ip	Structure	Analgetic[a] ED_{50}, mg/kg ip
3 (n = 0)	2.8 (2.2–3.6)	2 (n = 0)	105
3 (n = 2)	6.6 (4.7–9.4)	2 (n = 1)	0.059 (0.02–0.11)
4 (n = 0)	38 (24–44)	2 (n = 2)	0.0028 (0.0012–0.0064)
4 (n = 1)	4.8 (3.3–7.0)	2 (n = 3)	0.047[b] (0.032–0.068)
4 (n = 2)	0.35 (0.16–0.77)	6	2.8 (1.8–4.2)
4 (n = 3)	0.38 (0.17–0.84)	7	4.6 (2.7–9.1)
4 (n = 4)	0.8 (0.61–1.04)		
Morphine	1.3 (0.92–1.79)		

[a]Rat tail pressure. [b]Previously reported as 2.1 × morphine.[2]

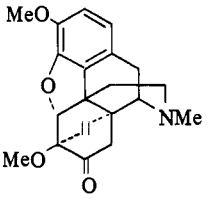
and benzyl and between benzyl and phenethyl; thereafter, marginal reductions are observed. The effect is similar to that found for the series of alcohols **1** and **2** and is in keeping with the postulate of a second lipophilic site on the analgetic receptor.[3]



1, R = Me
2, R = Ph



3, R = Me
4, R = Ph



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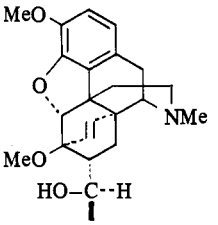

6, R = H
7, R = Et

Experimental Section

Melting points were determined with a Kofler hot-stage apparatus and are uncorrected. Where analyses are indicated only by symbols of the elements, the results obtained for those elements were within ±0.4% of the theoretical values. The structures of all compounds were assigned on the basis of compatible ir and nmr spectra.

6,14-*endo*-Etheno-7β-hydroxy-7α-phenyltetrahydrothebaine (4, n = 0) (General Procedure for Tertiary Alcohols of Structures 3 and 4). 6,14-*endo*-Etheno-7-oxotetrahydrothebaine[1] (5, 30 g) in C_6H_6 was slowly added to a stirred, boiling ethereal solution of PhMgBr [from Mg (0.6 g) and PhBr (4.0 g)]. The mixture was set aside at room temperature for 18 hr and was then poured with vigorous stirring into saturated aqueous NH_4Cl. The organic layer was collected and the aqueous solution washed with C_6H_6. The combined extracts were dried (Na_2SO_4) and evaporated. Crystallization of the residue from EtOH afforded 4 (n = 0) (0.9 g), mp 215–216°. *Anal.* ($C_{27}H_{29}NO_4$) C, H, N.

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Penicillinase Inhibition†

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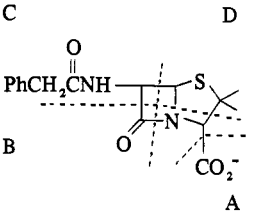
Penicillinase (EC 3.5.2.6), the enzyme catalyzing the hydrolysis of the β-lactam ring in penicillin, imparts resistance to the antimicrobial action of penicillin.[1] If a potent, yet safe, inhibitor of this enzyme could be found, it would be an effective adjunct in penicillin therapy against resistance common in many strains of staphylococci.

Many reports have dealt with penicillinase inhibition; notable among these are the early studies of Behrens and coworkers[2] and more recently those of Depue, *et al.*,[3] and Saz, *et al.*[4] Although these and other studies examined a wide range of compounds, no clinically significant inhibitors were uncovered. For this reason an analysis of the structural features important for penicillinase inhibition was undertaken.

The investigation of penicillinase inhibition described herein is directed toward delineation of the most important binding sites in the substrate, penicillin. Since penicillinase obtained from *Bacillus cereus* 569/H hydrolyzes benzylpenicillin, penicillin G was chosen as the model system for study. In Chart I, benzylpenicillin is dissected into regions

Chart I. Dissection of Benzylpenicillin into Possible Binding Sites



which might be expected to contribute to substrate binding.

Region A includes the carboxyl group. This moiety is included in all the compounds because (a) it is assumed to contribute substantially to the binding and (b) it imparts water solubility to all the test compounds.

Region B, the site at which hydrolysis actually occurs, comprises the lactam carbonyl group; the lactam nitrogen is not included in this region since it is not believed to

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Table I. Inhibitory Effects of Compounds Containing Regions A and B of Benzylpenicillin

No.	Compound	% inhibition[a]
1	*N*-Acetylamino acids[b]	0–3
2	*N*-Benzoylamino acids[c]	2–12
3	β-Benzoylpropionic acid	0
4	4-Acetylbutyric acid	0
5	*m*-Hydroxyphenoxyacetic acid	0
6	*p*-Fluorophenoxyacetic acid	0
7	*m*-Fluorophenylacetic acid	0
8	Succinimidoacetic acid	0
9	3-Nitropropionic acid	0
10	3-(2-Thenoyl)propionic acid	0
11	Nicotinic acid *N*-oxide	0
12	*m*-Fluorobenzoic acid	9
13	*o*-Hydroxyphenylacetic acid	4
14	*o*-Hydroxycinnamic acid	7
15	2-Pyridylacetic acid	4
16	3-Pyridylacetic acid	18
17	*o*-Nitrophenylacetic acid	18
18	*o*-Formylphenoxyacetic acid	10
19	3,4-Dihydroxyhydrocinnamic acid	15
20	4-(*p*-Nitrophenyl)butyric acid	20
21	γ-Hydroxybutyric acid	20
22	*o*-Fluorophenylacetic acid	15

[a] See Experimental Section for an explanation of these values. [b] Included among the amino acids are (a) L-alanine, (b) DL-leucine, (c) DL-valine, (d) DL-methionine, (e) glycine, (f) 4-aminobutyric acid, (g) DL-proline, (h) L-cysteine, (i) DL-phenylalanine, (j) DL-norleucine, and (k) DL-glutamic acid. [c] The presence of an indole moiety imparts exceptional inhibitory powers to a molecule. Whereas *N*-acetylamino acids 1 show virtually no inhibitory effect, *N*-acetyl-DL-tryptophan exhibited 11% inhibition. Whereas *N*-benzoylamino acids 2 produce inhibitory effects of less than 12%, *N*-benzoyl-DL-tryptophan exhibited 30% inhibition. Indoleacetic acid, indolepropionic acid, and indolebutyric acid exhibit inhibitions of 19, 8, and 28%, respectively, whereas 3-phenylpropionic acid shows only 2% inhibition. Included among the amino acids are (a) DL-valine, (b) DL-leucine, (c) DL-alanine, (d) DL-methionine, and L-glutamic acid.

greatly affect the electronic character of the carbonyl group.[5] Both of the prominent characteristics of the carbonyl group, the carbon–oxygen dipole and the hydrogen bonding ability of the oxygen, have been investigated.

Regions C and D are less distinctive in their electronic characteristics than are A and B. Region C includes the phenylacetamido group, which was tested as a single entity. Whatever contribution region D makes to enzyme-substrate binding is expected to arise from the van der Waals forces associated with hydrophobic interactions.

Region B analogs included in Table I are compounds containing a carboxyl group and another function which resembles the lactam carbonyl in that it possesses either a permanent dipole or hydrogen bonding capabilities or both. All of these compounds possess a strain-free conformation which allows the two groups to be positioned as a distance approximating that between the carboxylic acid and the lactam carbonyl in penicillin itself.

These compounds are generally poor inhibitors, and, in fact, it cannot be assumed that the limited inhibition observed in some cases is always due to the type of active site binding for which the compounds were chosen. For example, succinimidoacetic acid (**8**), which was tested because the position at which its carbonyl stretching frequency occurs in the infrared is the same as that of the lactam carbonyl in penicillin, suggesting similar electronic character for both carbonyl groups, shows no inhibition.

Region C. This moiety appears to make a definite contribution to the binding of penicillin to penicillinase (Table II),

Table II. Inhibitory Effects of Compounds Containing Regions A and C of Benzylpenicillin

No.	Compound	% inhibition
23	Phenaceturic acid	8
24	*N*-Phenylacetyl-5-aminovaleric acid	23
25	*N*-Phenylacetyl-4-aminobutyric acid	33
26	*N*-Phenaceturoyl-DL-valine	34
27	*N*-Phenaceturoyl-L-valine	20
28	6-Aminopenicillanic acid	78

since the ability of the substrate to bind to the enzyme is substantially reduced by removal of the phenylacetyl group. This is demonstrated in the case of 6-aminopenicillanic acid (**28**), which exhibits only 78% inhibition at a concentration 90-fold greater than penicillin.

Further evidence that the phenylacetamido group contributes to binding is illustrated by the fact that *N*-phenylacetyl-4-aminobutyric acid (**25**) and *N*-phenylacetyl-5-aminovaleric acid (**24**), in which the distance between the carboxyl group and phenylacetamido moiety approximates that of penicillin, inhibit the hydrolysis of penicillin by 33 and 23%, respectively. These results can be contrasted with the inhibitory effect of phenaceturic acid (**23**) (8% inhibition) in which the distance between the carboxyl group and the phenylacetamido residue is much less than in penicillin. That the phenyl group contributes to the binding is strikingly demonstrated by the fact that *N*-acetyl-4-aminobutyric acid (**1f**) shows no inhibition whatsoever.

Whereas *N*-acetylvaline (**1c**), which does not contain the phenylacetamido residue, shows no inhibition of penicillinase, *N*-phenaceturoyl-DL-valine (**26**) and *N*-phenaceturoyl-L-valine (**27**) show a moderate inhibitory effect (34 and 20%, respectively.)‡ The fact that the DL compound is a better inhibitor than the L compound is considered good evidence for binding of these compounds at the active site of the enzyme, since the asymmetric carbon atom bearing the carboxyl group in penicillin G has the D configuration.[6]

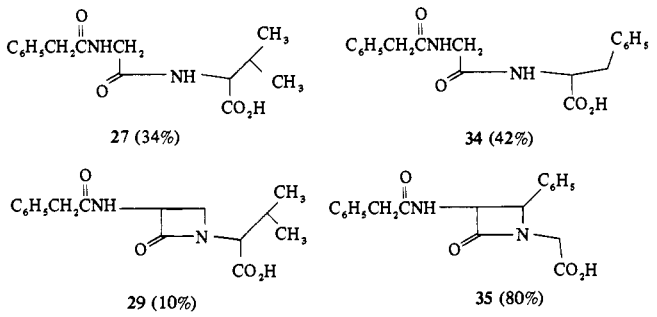
The fact that DL- and L-*N*-phenaceturoylvaline show no greater inhibition than *N*-phenylacetyl-4-aminobutyric acid or *N*-phenylacetyl-5-aminovaleric acid, which differ from the former in that the *N*-phenaceturoylvalines contain a region B carbonyl, supports the above conclusion that region B does not contribute significantly to binding.

Region D. This region of the penicillin nucleus appears to contribute less to binding than does region C. Compounds containing only a carbonyl group attached to a hydrophobic residue show essentially no inhibition. Thus butyric acid (**30**, 2%), 3-phenylpropionic acid (**31**, 0%), and *N*-acetyl-*S*-methyl-DL-penicillamine (**32**, 4%) exhibit little or no inhibitory effect. Furthermore, *N*-phenaceturoyl-*S*-methyl-DL-penicillamine (**33**), containing region D of benzylpenicillin in its entirety, manifests only 17% inhibition, which is probably due wholly to the contribution of the phenaceturoyl residue.

However, the possibility of a contribution of region D to binding cannot be discounted on the above evidence alone. The substitution into region D of a phenyl group, which might be expected to contribute to binding through hydrophobic interactions, enhances binding. This is demonstrated in Chart II, where the following comparisons are made: *N*-phenaceturoyl-DL-valine (**27**, 34%) *vs.* *N*-phenaceturoyl-DL-phenylalanine (**34**, 42%), and dethiobenzylpenicillin (**29**,

‡ It is surprising that dethiobenzylpenicillin (29), which shows only 10% inhibition and is not a substrate, is a poorer inhibitor than are the phenaceturoylvalines 26 and 27, since the former is expected to be more closely analogous to penicillin.

Chart II. Comparison of Inhibition Produced by Compounds Containing Regions A and C (27 and 29) with That of Compounds Possessing A, C, and D (34 and 35)



10%) *vs.* 1-(carboxymethyl)-3-phenylacetamido-4-phenyl-2-azetidinone (**35**, 80%). In each comparison between structurally similar compounds, the compound bearing a phenyl group is the better inhibitor.

All the inhibition values reported herein were determined at an inhibitor/substrate ratio of 90. The overall inhibition was disappointing; even compounds which are markedly similar to penicillin G, for example, dethiobenzylpenicillin (**29**) and *N*-phenaceturoyl-*S*-methyl-DL-penicillamine (**33**), are bound poorly by penicillinase derived from *B. cereus* 569/H.

Experimental Section

The penicillinase (Nutritional Biochemicals Corp., Cleveland, Ohio) used in this study was a purified from *B. cereus* 569/H. A solution of the enzyme was prepared by dissolving a 50,000-unit sample of the enzyme in 5 ml of 0.5% aqueous gelatin (Pharmagel B, Pharmagel Corp., N. Y.), which imparts stability to the enzyme solution.[7]

This solution was refrigerated at all times. Enzyme stock solutions were prepared fresh daily by diluting 0.25 ml of the above solution with 500 ml of 0.5% gelatin solution. The substrate stock solution, prepared fresh daily, was a 0.188 *M* aqueous solution of the potassium salt of penicillin G (Sigma Chemical Co., St. Louis, Mo.). All inhibitor stock solutions were adjusted to pH 7 with dilute sodium hydroxide and were 0.424 *M* in inhibitor. All inhibitors except *N*-phenaceturoyl-DL-phenylalanine (**34**) have been previously described and were either commercially available or prepared by conventional methods. We are grateful to Dr. J. N. Wells of Purdue University for the sample of 1-(carboxymethyl)-3-phenylacetamido-4-phenyl-2-azetidinone, whose synthesis has been previously described.[8] The phenaceturoyl derivatives were synthesized by condensation of phenaceturoyl azide with the corresponding amino acid as illustrated in the following procedure. A pH-stat method similar to that reported by Zyk and Citri[9] was employed in this work. The pH-stat titrations were carried out at 25° at pH 6.93 using a Radiometer (Copenhagen, Denmark) Titrator II in conjunction with the following Radiometer equipment: Auto-Burette Type ABU 1b, pH Meter 26, and Titrigraph SBR2c. All inhibition studies were performed in the same manner. Enzyme solution (10 ml) and 2 ml of inhibitor solution, or water in the case of the standardization assay, were pipetted into the reaction vessel. After this solution had been adjusted to pH 7.00, 0.050 ml of substrate solution was added; thus, the ratio of inhibitor to substrate was 90. The pH-stat titration was then carried out with 0.0100 *M* sodium hydroxide up to 0.25 ml of base, so that the hydrolysis of penicillin was monitored up to 27% of completion. Under these conditions, base uptake gave a linear plot against time, and no product inhibition was detected. By this method the stoichemistry of the hydrolysis reaction was within 3% of the theoretical value; the reproducibility of the inhibition results was uniformly better. Substrate activity could not be detected in any of the inhibitors tested.

N-Phenaceturoyl-DL-phenylalanine (**34**). To a solution of 1.98 g (12 mmol) of sodium hydroxide was added 2.65 g (12 mmol) of phenaceturoyl azide. The mixture was stirred for 6 hr at room temperature and then filtered. The filtrate was acidified with hydrochloric acid and again filtered to give 1.6 g (39%) of a solid, mp 166–169°. Recrystallization from acetone yielded *N*-phenaceturoyl-DL-phenylalanine (**34**) as a white solid, mp 178–180°.

Anal. Calcd for $C_{19}H_{20}N_2O_4$: C, 67.04; H, 5.92; N, 8.23. Found: C, 66.85; H, 6.16; N, 8.31.

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Benzopyrones. 9.[1] Synthesis and Pharmacology of Some Novel Bischromones

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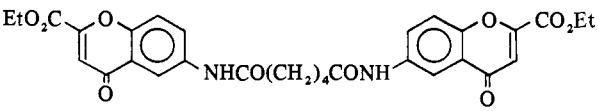
The antiallergic action of bischromones is of longer duration than that of their simpler analogs.[2] A number of such compounds in which the two chromone moieties are joined together through an alkyl or alkoxy linkage are described in the literature.[3,4] We now report the synthesis of several bischromones in which the two units are linked through amide groups attached to C-2, C-6, or C-8 and one in which the internuclear bridge contains an amide and an ether linkage. Both kinds of linkages are resistant to cleavage and therefore would be expected to promote a long duration of action.

Chemistry. Compounds **1** and **2** in which the amino group



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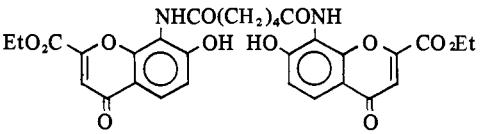

2

is attached to the chromone ring were prepared by reacting adipoyl chloride with ethyl 6-amino-4-oxo-4*H*-1-benzopyran-2-carboxylate[5] and ethyl 8-amino-7-hydroxy-4-oxo-4*H*-1-benzopyran-2-carboxylate,[6] respectively. Both products are high-melting compounds of very low solubility in commonly used solvents and could not be recrystallized. Their insolubility also precluded their hydrolysis to the carboxylic