Tetrahedron. Vol. 30, pp. 3677 to 3688. Pergamon Press 1974. Printed in Great Britain

PEPTIDE SYNTHESIS WITH BENZISOXAZOLIUM SALTS—I

PROPERTIES OF SUBSTITUTED 2-ETHYL-BENZISOXAZOLIUM SALTS

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(*Received in the USA* 7 *June* 1974; *Received in the UK for publication* 17 *June* 1974)

Abstract—The general features and limitations of 2-ethylbenzisoxazolium fluoroborate as a peptide coupling reagent are described. Strategies for effecting improvements through benzo substitution led to the preparation and study of the 5-nitro, 7-hydroxy, 5-nitro-7-hydroxy, and 4,6-dihydroxy-2-ethylbenzisoxazolium cations. The general properties of these and of several related systems are outlined.

In the period since the reinvestigation by Woodward and Olofson of the properties of Claisen and Mumm's isoxazolium salts, considerable attention has been devoted to the design of substituted isoxazolium salts which can be used advantageously as peptide coupling reagents.[1] As we have noted previously,[2,3] though qualitatively similar to that of simple isoxazolium salts, the chemistry of 2-alkylbenzisoxazolium salts shows striking quantitative differences which require that the applications of these reagents to amide formation be explored independently, without a likelihood of supporting analogy between the two compound classes. In this paper we describe the chemistry of amide formation using benzisoxazolium salts, together with the results of our attempts to overcome the deficiencies of the 2-ethylbenzisoxazolium cation by the synthesis of derivatives, modified by substitution in the benzo positions. The most successful amide-forming reagent which has resulted from our attempts is the 2 - ethyl - 7 - hydroxybenzisoxazolium cation; optimum conditions for use of this reagent, mechanistic aspects of its action, and results of its application to peptide synthesis are presented in the accompanying two papers.

1. *General points*

As a result of our earlier work, the 2-ethylbenzisoxazolium cation has been shown by indirect, kinetic arguments to react with carboxylate anions by the mechanism of Scheme I, for which only the starting materials and products can be observed directly, intermediates **2** and **3** being too rapidly decomposed for observation. The product **4** is a phenolic ester, and the unique feature of any of the isoxazole coupling reagents is a reaction sequence which cleanly and efficiently converts a carboxylic acid or its anion to a phenol or enol ester. Like the more conventional routes to these esters, the above reaction sequence involves an overactivated, racemization-prone intermediate, the iminoanhydride, **3**, and in large part, the virtue of the benzisoxazole route to activated esters rests on the relative rate advantage which the intramolecular acyl transfer, **3**→**4**, has over other intramolecular processes such as oxazolone formation or asparagine isoimide formation.

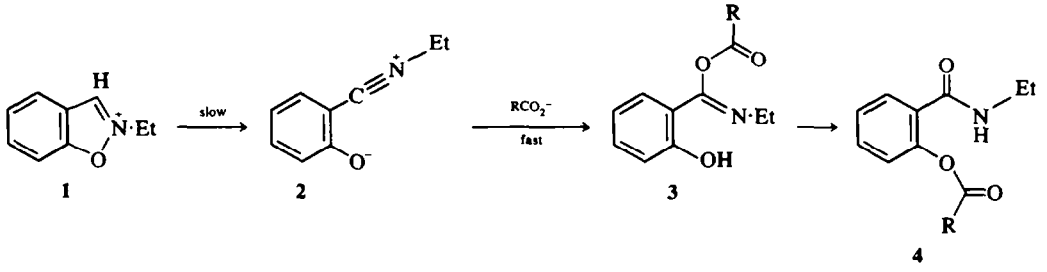
The general features of peptide coupling with a given benzisoxazolium salt can be addressed in terms of three sets of questions:

1. *Activation conditions* (**2**→**4**). Under what condition does the benzoketoketenimine, **2**, react cleanly with carboxylic acids? What are optimum yields, and what by-products are formed?

2. *Acyl transfer step* (**3**→**4**). Under what conditions does this transfer occur cleanly, without competing reactions of the carbonyl of **3** with internal or external nucleophiles?

3. *Activated ester* **4**. Are the esters **4** stable to storage? How rapidly and cleanly do they react with peptide amine nucleophiles? Do they discriminate effectively between water or alcohols and amine nucleophiles? What byproducts may be expected from peptide couplings with these esters? How much racemization attends their use?

Throughout discussion of the examples of this paper we rely on the behavior of the 2-ethylbenzisoxazolium cation as a reference point, and we assume that the mechanistic details which have been demonstrated for this system apply as well to substituted derivatives of it. This assump-



tion seems particularly reasonable in light of the retention of the general features of this mechanism through the very substantial perturbation of properties of intermediates which results from change of an isoxazolium to a benzisoxazolium system.

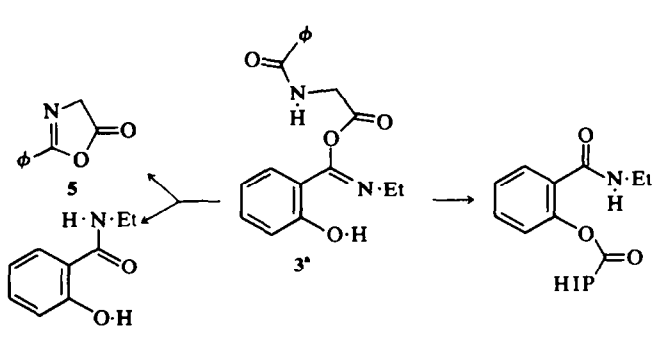
2. *Properties of the 2-ethylbenzisoxazolium cation as a peptide coupling reagent*

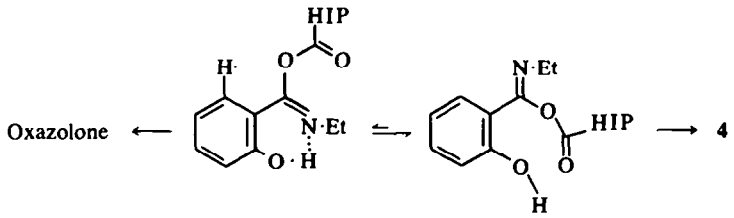
Previously reported work[2,3] established that **1** reacts with simple carboxylic acids in aqueous solution at pH 5–5·5 to give 85–90% yields of ester, **4**. With 0·01 M acetate ion, the yield of ester is 80%, and even with 0·001 M acetate ion, the estimated ester yield is 30%, the remaining product being phenol. The reaction has approximate halftimes at pHs 4 and 5 of 2 min, and 15 sec, respectively. For optimum activation results, the pH must be high enough to convert the acid to its anion which is its reactive form, yet must not greatly exceed 5, to avoid increased formation of a pale yellow polymeric material, believed to result from reactions of high local concentrations of the benzoketoketenimine, **2**. Although conversion of acids to ester **4** was also observed if a triethylamine-carboxylic acid salt was combined with **1** in organic solvents such as dichloromethane or acetonitrile, the yields were consistently lower than with the aqueous preparation, and contamination with yellow polymer was much more serious.

Hippuric acid has been noted[2] to react with **1** under the above conditions to yield a mixture of 2-phenyloxazol-5-one as well as the expected active ester, a result which indicates that for this system, intramolecular oxazolone formation can compete effectively with the desired acyl transfer. A disposition of the system toward this result doubtless is related to the relative stability of conformers **x** and **y** of the hippuryl iminoanhydride, **3**[a]. The transition state leading to oxazolone can be stabilized by the strong hydrogen bond of the salicylaldimine moiety of **x**, while the transition state for acyl transfer is deprived of internal H-bonding. In fact, a variety of dipeptide acids have been observed to yield active ester oxazolone mixtures when subjected to simple aqueous activations, and it is evident that under these conditions, **1** is unsuitable for peptide applications.

The possibility of altering the reaction preferences of **3** by a change of reaction conditions was made real by the implications of the data of Table 1, which also independently establish the intermediacy of a highly activated, transient acyl species which can form either oxazolone or activated ester.

The results establish that the product ratio is sensitive to the order of combination of reagents and that if **1** is added slowly to the carboxylate salt, active ester is the substantial product. Experiments 3, and 5, establish the remarkable result that the product ratio depends on the rate of addition of **1** *only if* **1** *is added in excess of the amount of carboxylate.* The product ratio must therefore depend on conditions which result after the initial reaction of **1** with salt is substantially complete and this result can be rationalized only if an intermediate is formed whose lifetime is of the timescale of the rapid additions (*ca* 1 sec) and whose decomposition path is determined by the differing final reaction conditions of experiments 3 and 5, i.e., 0·2 equiv of **1** in the latter case, and 0·1 equiv of triethylammonium carboxylate in the former. The intermediate therefore yields oxazolone under





3[a]

Table 1.[a] Reactions of 1 with ZGlyPheOH and Et_3N in Acetonitrile

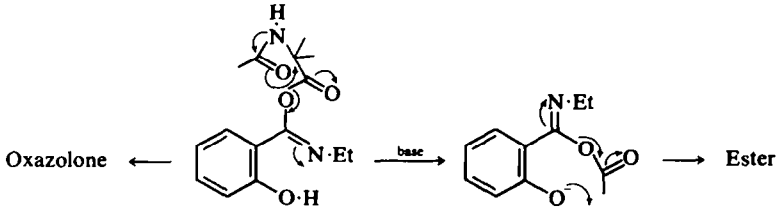
Reaction conditions	Oxazolone yield[a]	Ester yield[a]
1. Acid (ZGlyPheOH) + Amine (Et_3N) 1·0 eq 1·0 eq added to 1 (1·1 eq)	90%	10%
2. 1 (0·9) added slowly (~ 1 min) to Acid (1·0) + Amine (1·0)	0	80
3. 1 (0·9) added rapidly (0·5 sec) to Acid (1·0) + Amine (1·0)	1	78
4. 1 (1·2) added slowly as in 2. to Acid (1·0) + Amine (1·0)	3	78
5. 1 (1·2) added rapidly as in 3. to Acid (1·0) + Amine (1·0)	65	20

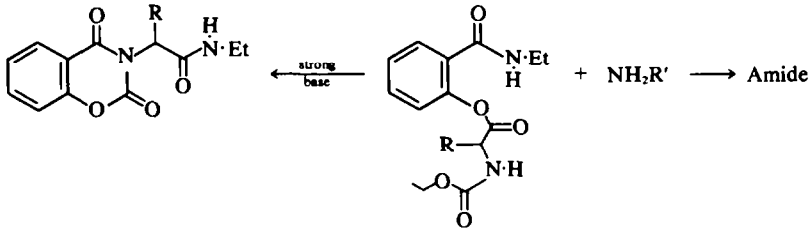
[a]Yields for the ester were obtained by isolation, and spectrometrically, at 1770 cm^{-1}; the oxazolone yield was obtained spectrometrically, at 1830 cm^{-1}.

neutral or acidic and active ester under mildly basic conditions, a reasonable result if the internal acyl shift from 3 occurs only with its conjugate base.

Since the anhydride 3 appears to have sufficient lifetime to equilibrate between phases of a heterogeneous aqueous activation, the above results suggest addition of a nonnucleophilic base of pK_a *ca* 5 which distributes between water and organic solvents. In fact, the use of aqueous pyridine buffers, overlayered with dichloromethane or ethyl acetate, results in suppression of the oxazolone pathway and a significant increase in yield, typified by the formation of the recrystallized active ester of ZGlyPheOH in a yield of 95–97%. Similar activations with eleven simple amino acid derivatives gave an average yield of 92% (range, 88–97%); ten di or tripeptide acids were converted to active esters in yields of 73–98%, average 88%; and for the three acids: $Z(GlyLeuGly)_2OH$, $Z(GlyLeuGly)_4OH$, $Z(GlyLeuGly)_8OH$, the average active ester yield was 70% (with a range of 61–90%).[5] Although histidine and arginine derivatives have not been explored, C-terminal asparagine or glutamine acids give no evidence of amide participation, and the steric effects of valine or proline do not interfere. The acylsalicylamide esters, 4, are generally readily crystalline substances which are stable to storage.

The above procedure implies that optically pure peptide esters of structure 4 are a readily accessible class of substances; unfortunately, though a useful class of acylating agents, these substances fall short of the ideal in several critical regards. Although they appear to react cleanly with peptide amines in dipolar aprotic solvents yielding amides and N-ethylsalicylamide, which can readily be removed by extraction with alkali or solution in carbon tetrachloride, the reactions are slow, occuring at roughly a twentieth the rate of the corresponding p-nitrophenyl ester coupling,[6] by-products resulting from Brenner rearrangements occur in the presence of strongly basic amines such as triethylamine,[7] and

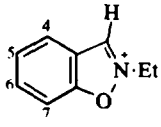




1–2% of racemate is formed during the formation of ZGlyPheGlyOEt.[8]

Despite these limitations, we have used these esters for the synthesis of roughly twenty simple peptide derivatives, combining active ester with amine in acetonitrile or DMF overnight. The byproduct, N-ethylsalicylamide, can be easily removed by alkali extraction or in the case of highly crystalline, insoluble products, by extraction with carbon tetrachloride or ether, in which it is very soluble. Yields averaged 85–90%. For large scale conversion of acids to amides, the method is very convenient and practical, although it cannot be expected to give satisfactory results in highly hindered cases.

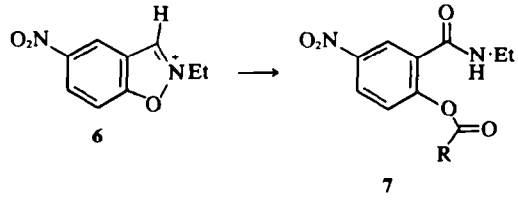
Four unsolved problems are thus posed by this system, two major, two minor. The minor problems are the Brenner rearrangement observed in the presence of strongly basic amines, and the presumably narrow margin which arises, even with pyridine buffering, between the desired rearrangement of **3** to active ester, and the unimolecular reaction of **3** with internal peptide nucleophiles, typified by oxazolone formation. The major problems are the unsatisfactory racemization level and the slow coupling rates. The strategy which led to examination of the examples of this paper was the



construction of derivatives of **1** in which substituents in the benzo ring positions have been chosen to ameliorate one or more of the above problems.

3. *5-Nitro and 4,6-dihydroxy-2-ethylbenzisoxazolium salts*

An obvious choice for a solution of the problem of insufficient active ester reactivity is the 5-nitro-2-ethylbenzisoxazolium cation, prepared earlier for mechanistic study.[3a] Unfortunately, reactions of **6** with carboxylic acids to form nitroacylsalicylamides, **7** could not be induced to occur in satisfactory yields. Although the aqueous pyridine conditions proved more satisfactory than nonaqueous, the yield for ZGlyOH was at best 33%, while



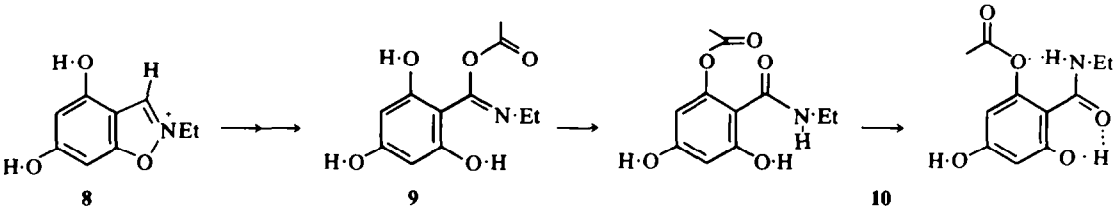
6 **7**

no reaction was observed with hippuric acid. Extensive polymer formation accompanied all attempted activations. Similar problems probably account in part for the low yields observed by Rajappa and coworkers for peptide coupling reactions carried out with **6** and with 5,7 - dichloro - 2 - ethylbenzisoxazolium fluoroorate.[3b]

Although one can explain these results by noting that **6** reacts 10^4 times more rapidly with bases than **1** and the resulting benzoketoketenimine must be present at high local concentrations which would favor polymerization, an alternative speculation is that polymerization requires the neutral ketoketenimine while reaction with nucleophiles require its conjugate acid; nitro substitution should make the latter more acidic, favoring polymerization at a given pH. In any event, it is clear that simple modification by the introduction of strongly electron withdrawing groups seriously interferes with the cleanness of the activation steps.

A second system of interest is the 4,6-dihydroxy-2-ethylbenzisoxazolium cation, readily available from phloroglucinaldehyde. It was hoped that the intermediate, **9**, would show no tendency for intramolecular decomposition other than the internal acyl transfer which is made favorable by the hydrogen bond to the 2-hydroxyl which orients the acyl function for transfer to the 6-hydroxyl. It was further hoped that the active ester, **10**, would show increased reactivity over **4** as a result of a stabilization effect of the 6-hydroxyl on an incipient 2-oxyanion.[9]

Although **8** was highly hygroscopic and could not be easily purified, crude preparations could be used to obtain active esters of ZGlyOH (92%) and ZGlyPhe OH (86%), by means of the aqueous pyridine procedure. Reaction of **8** with triethylammonium hippurate in acetonitrile under any of the conditions of Table 1 gave solely active ester; no oxazolone could be detected. Although amides were obtained in good yield by reaction of **10** with



8 9 10

amines, no significant increase in reactivity over the corresponding esters, **4**, was noted, and a ready racemization (20% in 15 min) was observed when the active ester of ZGlyPheOH was contacted with triethylamine in DMF. Not surprisingly, the esters, **10**, were somewhat difficult to purify, and the phloroglucinamide byproduct was prone to oxidation. Thus, although this compound sequence demonstrated the feasibility of rendering the iminoanhydride acyl transfer more favorable, the system offered no other advantage over the more available and tractable parent system.

4. *7-Hydroxy-2-ethylbenzisoxazolium salts*

Since increase of active ester reactivity through introduction of strongly electron-withdrawing groups results in unsatisfactory activation chemistry, the hope for increasing the activity of benzisoxazole-derived active esters must lie in the introduction of groups which can catalyze the aminolysis reactions of phenolic esters without increasing their thermodynamic activation. Exactly such an effect is needed to resolve the racemization problem as well. The work of Hansen and Bender on the hydrolysis of catechol half esters[10] suggested consideration of the 7-hydroxy-2-ethylbenzisoxazolium cation, **11**.[11] Similar expectations of a selective catalysis of ester aminolysis led Young and coworkers to an independent formulation of this problem[12] and an exploration of the properties of amino acid-catechol half esters.[13]

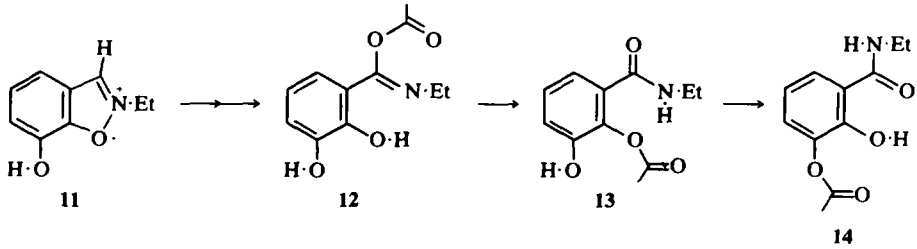
The salt, **11**, was prepared in 78% yield from 2,3-dihydroxybenzaldehyde, available in turn from *o*-vanillin by treatment with HBr. Reaction with carboxylic acids was found to be most conveniently carried out using the pyridine-water procedure, with careful adjustment of pH to the range 4·5–5·0. Yields with simple carboxylic acid derivatives lay in the range of 70–80%; as indicated in the accompanying papers, peptide acids give better results. The reaction has a half time at pH 4·5, 22°, of *ca* 1–2 min. Although somewhat more yellow polymeric byproducts accompanied these activations than those of the parent system, **1**, in most cases, crystalline product could be isolated in high yield without difficulty.

In principle, either **13** or **14** could be expected as products of this activation sequence; **14** is likely to be more stable by virtue of its *meta* acyloxy substitution[14] and its strong intramolecular H-bond. Since 5-ring acyl migrations are known to be very rapid,[15] one expects to isolate **14** as the product of this activation sequence.

That **14** is in fact formed follows from a variety of independent lines of evidence. When the acetate ester derived from **11** is treated with diazomethane in the presence of a catalytic amount of fluoroboric acid,[16] the resulting product can be hydrolyzed to a methoxyhydroxy - N - ethylbenzamide which is distinct from 3 - methoxy - 2 - hydroxy - N - ethylbenzamide, prepared by hydrolysis of 7 - methoxy - 2 - ethylbenzisoxazolium fluoroborate, and which can be formulated spectroscopically as 3-hydroxy-2-methoxy - N - ethylbenzamide. Since the 2 and 3-acetoxy esters must be in rapid equilibrium, it could be argued that the product of methylation may be a result of a kinetic preference of diazomethane, and therefore not of structural significance. However, sterically hindered, strongly H-bonded phenols usually react sluggishly and incompletely with diazomethane, and we therefore regard the isolation of 2-methoxy product as indicative of an equilibrium, **13**⇄**14**, which strongly favors the latter.

Since the UV contribution of an acetoxy group to a phenyl function is usually very small, the absorption spectrum of **14** is expected to resemble that of N-ethylsalicylamide, while that of **13** should be modeled by *m*-hydroxybenzamide. Table 2 presents these data.

A structural tool of unique power for the



11 12 13 14

Table 2. UV spectra of **14** R=CH_3, and models

	pH	uv max, H_2O, nm (ϵ)
Acetate product (**14**, R=CH_3)	1–5	299 (3800), 238 (8100)
	8	326 (6500)
N-Ethylsalicylamide	1–7	296 (3600), 240 (8200)
	12	325 (6100)
m-Hydroxy-N-ethylbenzamide	1–7	285 (2190)
	12	310 (2830)

structural assignments of phenolic isomers is near IR spectroscopy, utilized many years ago by Pauling and others.[17] Thus in dichloromethane solution, a secondary amide N-H generally shows overtone absorption in the region 1·46–1·50 μ, a non-H-bonded phenol absorbs in the range 1·41–1·43 μ with an extinction coefficient of 1·5–3·0, and H-bonding shifts a phenol absorption maximum to 1·44–1·47 μ, with a marked decrease in extinction coefficient. Chelated phenol O–H generally shows no detectable absorption in this region. Table 3 presents data for the acetate product and related models; it may be noted that the spectrum of 3 - methoxy - 2 - hydroxy - N - ethylbenzamide exactly matches that of the product.

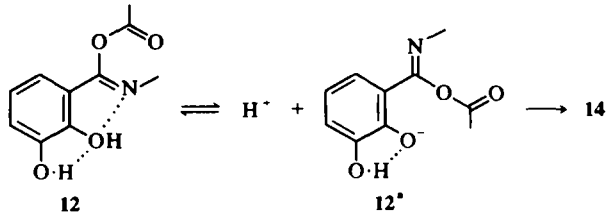
Although no instrumental study at short time spans has been conducted, we have never observed results from a rapid product workup which indicate the presence of significant amounts of the intermediate, **13**. As with **1**→**4**, the activation sequence **11**→**14** appears to occur very rapidly and cleanly, without observable intermediates.

sensitive test of this point was made possible as a result of the very small racemizing tendency of the esters **14**.[11] When ZGly-L-PheOH and triethylamine in acetonitrile was added slowly to a stirred solution of **11** (conditions which with **1** yield 90% oxazolone), addition of GlyOEt in DMF at 0° yielded 75% tripeptide, of which 1·0% was racemic. More practically, when the activation was carried out in aqueous pyridine and the neutral product fraction subjected to GlyOEt in cold DMF without purification, 80% of tripeptide was obtained, 0·03% of which was racemic. Since the pure active ester, **14**, of ZGly-L-PheOH is observed to yield 0·010–0·015% of racemate under these conditions,[11] one can conclude that under the normal aqueous activating conditions as much as 0·02% oxazolone might be formed from the iminoanhydride, **12**. It seems likely that the acidity of the catechol moiety of **12** is increased over that of the phenol of **3** and the greater abundance of the anion **12**^{a} at pH 5 explains this favorable result.

The 7-hydroxybenzisoxazolium system satisfactorily solves all four of the problems posed by the system, **1**. In earlier reports we have outlined the rapid amine acylations which the active esters **14** undergo at basicities sufficient to ionize the

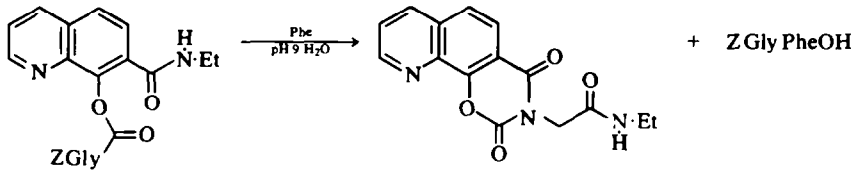
Table 3. Near IR data for the acetate product, **14**, R=CH_3 and models (CH_2CL_2)

Substance	Absorption, $\mu(\epsilon)$	Comments
N,N-Dimethylsalicylamide	no absorption	chelated O–H
N-Ethylsalicylamide	1·484 (0·60)	N–H; chelated OH
2,3-Dihydroxy-N-ethylbenzamide	1·485 (0·86)	N–H
	1·460 (1·07)	weakly H-bonded OH
2-Methoxy-2-hydroxy-N-ethylbenzamide	1·506 (0·34)	H-bonded N–H
	1·460 (0·63)	weakly H-bonded OH
3-Methoxy-2-hydroxy-N-ethylbenzamide	1·484 (0·60)	N–H
Acetate product = **14**	1·485 (0·62)	N–H



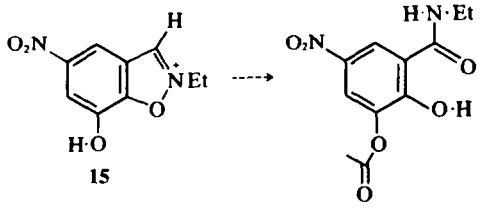
12 **12**^{a}

phenolic hydroxyl. A detailed presentation of data for the activation reactions of **11** and the coupling reactions of **14** are presented in the accompanying papers. Here we draw attention to a comparison of this 7-hydroxybenzisoxazole-derived system with the parent system. The Brenner rearrangement which is a minor problem with esters IV has as a structural requirement a 2-acyloxybenzamide function; it does not occur with the esters **14**. The iminoanhydride acyl partitioning has been shown to be much more favorable with **12** than with **3** (although under optimal conditions it need not be a serious complication with either system). The esters **14** rank among the least racemizing of the acylating agents known to the peptide chemist, and under basic conditions these esters are comparable to p-nitrophenyl esters in reactivity. This system therefore stands as our most successful attempt to apply the chemistry of the benzisoxazolium framework to peptide acylation problems.



17 18

It remains to describe some less successful attempts to exploit the principle of internally catalyzed aminolysis. The 7 - hydroxy - 5 - nitro - 2 - ethylbenzisoxazolium cation, **15** was prepared in several steps from 2,3-dihydroxybenzaldehyde (Experimental), but was found to react with carboxylic acids to give esters, **16**, in only low yields (25–40%), contaminated with polymer. The



15 16

ester **16** derived from ZGly-L-PheOH has been observed to couple in DMF with two equivalents of GlyOEt at 22° to give a 94% yield of tripeptide of which 0·017% was racemic. Although these esters couple rapidly in the presence of an equivalent of mild base and give the most favorable racemization results under these conditions of any method we have tested, we have been unable to develop a satisfactory route to them. Still less satisfactory were experiments in which **11** was treated with fuming sulfuric acid or sulfonyl chloride to give 7 - hydroxy - 2 - ethylbenzisoxazolium cations with sulfonate or chlorine in the 4 or 6 positions (structures not established). Couplings of ZGly-L-PheOH with GlyOEt gave racemization levels in the inexplicably high range of 0·2–2%, and further investigation of these systems was discontinued.

Along with the catechol half esters, esters of 8-hydroxyquinoline are believed to undergo assisted aminolysis,[16] and it was of interest to prepare the ester, **17**, which could be formed from a hypothetical isoxazolium salt. Unfortunately, the Brenner reaction tendency of this system proved to be very high, and attempted aminolysis of the ZGlyOH ester **17** gave significant amounts of a product assigned structure **18**, based on empirical formula and spectroscopic properties. This problem was far more serious with this ester than with the corresponding ester **4**, and the system was abandoned.

5. *Summary*

It seems likely that several problems which have appeared in the cases here considered will prove to be general for other attempts to tailor the properties of benzisoxazolium salts through benzosubstitution. Because electron-withdrawing substituents appear to result in unsatisfactory activation yields, some more subtle means of enhancing aminolytic reactivity must be resorted to. Retention of the 2-acyloxybenzamide structure of the simple esters, **4** runs a risk of Brenner-type side products. The introduction of a 7-hydroxyl group obviates these problems and results in a 3-acyloxybenzamide of lower thermodynamic activation. Its reactivity must rest on the catalytic effect of a 2-oxyanion, which necessitates basic aminolysis conditions. The properties of esters **16** suggest that further substitution can result in increased reactivity at lower pH with less racemization, and if there is further chemistry to be developed in this area, we believe it lies along these lines.

A different and fundamental question must be raised concerning further elaborations of the benzisoxazole system. Complex molecules such as these are costly, and presumably they can be considered realistically for peptide synthesis only if they yield outstanding results when applied to the couplings of large peptide fragments. Yet it is exactly with large molecule couplings, with their usual problems of insolubility, low reagent concent-

rations and end group inaccessibility, that reagents such as these, with so many intermediates between starting materials and products, are put to their most severe tests. For these reasons it seems likely that a more profitable direction for exploratory research lies not in a search for amide-forming reagents which can seek out and activate the rather inert carboxylate groups of a large molecule, but rather in the devising of selectively protected carboxyl moieties which can be carried through a synthesis and unmasked as activated carboxyls at its later steps. The simplest existing model of such a grouping is probably the BOC hydrazide functionality.[19]

EXPERIMENTAL

Solvents and reagents were reagent grade, and anhydrous $MgSO_4$ was used for drying; unless otherwise specified. IR spectra were determined with a Perkin-Elmer 237 spectrometer; NMR spectra, with Varian A-60 and T-60 spectrometers, using tetramethylsilane as internal standard; UV spectra, with a Cary 14 spectrometer; and near IR spectra, with a Zeiss PMQ II spectrometer, equipped with PbS detector. IR, NMR, and UV spectra are reported in units of cm^{-1}, δ, and nm, respectively. Elemental analyses were performed by Scandinavian Microanalytical Laboratory.

Benzisoxazoles. The benzisoxazoles of Table 4 were prepared from the corresponding salicylaldehyde by the method previously described;[2,20] substances marked with an asterisk were prepared using water rather than ethanol as solvent.

pyridine complex, prepared by adding 25·9 g (0·33 mole) distilled acetyl chloride to 27 ml cold, stirred pyridine, was added with the help of 150 ml dry pyridine to a stirred soln of 414 g (0·30 mole) of 2,3-dihydroxybenzaldehyde in 50 ml pyridine. After 1 hr at 20° the soln was refluxed for 3 hr, cooled, filtered, and concentrated. The residue was taken up in 300 ml CH_2Cl_2 and extracted with 6 × 100 ml 0·5 N HCl, washed with brine, dried, and evaporated. Recrystallization from hexane gave 50·9 g, 2 - hydroxy - 3 - acetoxybenzaldehyde, 94·3%, plates, m.p. 95–96°. (Found: C, 59·84; H, 4·67. $C_9H_8O_4$ requires: C, 60·00; H, 4·48).

To a cooled (10–15°) soln of 60 g 2 - hydroxy - 3 - acetoxybenzaldehyde in 600 ml HOAc was added over 30 min a mixture of 110 ml 70% HNO_3 and 60 ml H_2SO_4. After a further 30 min of stirring at 0°, the ppt was collected, washed with ether, and recrystallized from EtoAc-ether to give 59 g, 79%, needles of 2 - hydroxy - 3 - acetoxy - 5 - nitrobenzaldehyde, m.p. 175–177°.

A solution of 17·7 g nitroaldehyde in 350 ml EtOH and 300 ml H_2O was refluxed for 4 hr; cooling to 0° gave crystals which were recrystallized from EtOAc ether to give 13·2 g, 99%, needles, m.p. 230–233° dec; NMR (DMSO): 7·8 (d, 1, J = 3), 8·0 (d, 1, J = 3), 10·1 (s, 1). (Found: C, 45·72; H, 2·69; N, 7·80. $C_7H_5NO_5$ requires: C, 45·91; H, 2·75; N, 7·65).

Benzisoxazolium salts

General procedure. Alkylation with triethyloxonium fluoroborate was carried out in CH_2Cl_2 as described previously for the parent system.[2] With the insoluble 4,6-dihydroxybenzisoxazole, a small amount of acetonitrile was added to the solvent, and the mixture was filtered after 1 hr to remove unchanged amine. The 5-nitro and

Table 4. Properties and analytical data for benzisoxazoles

Substitution	Yield (%)	m.p.	Solvent for crystallization		Analysis C	H	N
4,6-Dihydroxy*	91	sh 216 246°dec	CH_3CN		55·63	3·61	9·26
				$C_7H_5NO_3$ requires:	55·63	3·34	9·27
7-Methoxy[21]	82	b.p. 64–65°/0·01 mm					
7-Methyl*	72	b.p. 36–38°/0·02 mm					
				(NMR (CCl_4): 2·5 (3,s), 619–715 (3,m), 8·6 (1,s)			
5-Nitro-7-hydroxy*	90	195–196°	CH_3CN		46·64	2·21	15·49
				$C_7H_4N_2O_4$ requires:	46·68	2·24	15·55
5-Nitro-7-methoxy	70				49·50	3·09	14·46
				$C_8H_6N_2O_4$ requires:	49·49	3·12	14·43

Salicylaldehydes. The 3-methylsalicylaldehyde was prepared in 10% yield from *o*-cresol as described by Tiemann and Schotten.[22] Phloroglucinaldehyde was prepared from phloroglucinol, following the procedure of Malkin and Nierenstein.[23] The preparation of 2,3-dihydroxybenzaldehyde and its conversion to 2 - ethyl - 7 - hydroxybenzisoxazolium fluoroborate is described in the accompanying paper; 2 - hydroxy - 3 - methoxy - 5 - nitrobenzaldehyde[24] was prepared by nitration of *o*-vanillin.

2,3-*Dihydroxy-5-nitrobenzaldehyde.* Acetyl chloride-

unsubstituted 2-ethylbenzisoxazolium fluoroborates were prepared as described previously.[2,3]

2 - *Ethyl* - 7 - *hydroxybenzisoxazolium* - *sulfonate* (**4** *or* **6**). Fuming 30% H_2SO_4, (11·7 ml; 0·08 moles), was added to 2 - ethyl - 7 - hydroxy - N - ethylbenzisoxazolium fluoroborate (18·8 g; 0·073 moles). After 24 hr, 400 ml ether was added, and after 2 hr the white ppt was collected and washed repeatedly with ether. The resulting solid was slurried in hot acetonitrile, collected, and recrystallized from hexafluoroisopropanol-ether to give 16·1 g, 90%, of zwitterion, m.p. 221–223° (dec). (Found: C,

Table 5. Properties and analytical data for substituted 2-ethylbenzisoxazolium fluoroborates

Substitution	Yield (%)	m.p.	Solvent for crystallization		Analysis C	H	N
4,6-Dihydroxy (**8**)	75	125–131° hygroscopic!	CH_3CN–EtOAc–CH_2Cl_2		not analyzed		
7-Methoxy	74	108–110°	CH_2CL_2–CH_3CN		45·45	4·56	5·08
				$C_{10}H_{12}NO_2BF_4$ requires:	45·22	4·57	5·29
7-Methyl	74	91–93° hygroscopic	CH_3CN–EtOAc		48·41	4·95	5·61
				$C_{10}H_{12}NOBF_4$ requires:	48·23	4·86	5·63
5-Nitro-7-hydroxy (**15**)	90	156–158°	CH_3CN–EtOAc		36·30	3·33	9·53
				$C_9H_9N_2O_4BF_4$ requires:	36·52	3·06	9·45
5-Nitro-7-methoxy	80	152–153°	CH_3CN–EtOAc		38·95	3·51	9·04
				$C_{10}H_{11}N_2O_4BF_4$ requires:	38·74	3·58	9·04

44·41; H, 3·66; N, 5·65; S, 12·84. $C_9H_9NO_5S$ requires: C, 44·44; H, 3·73; N, 5·76; S, 13·18); NMR (hexafluoroisopropanol): 1·6 (t, 3, J = 7), 7·2 (D, 1, J = 10), 7·7 (d, 1, J = 10), 9·4 (s, 1).

2 - *Ethyl* - (4 *or* 6) - *chloro* - 7 - *hydroxybenzisoxazolium fluoroborate.* A slurry of 2 - ethyl - 7 - hydroxybenzisoxazolium fluoroborate (9·4 g) in 50 ml SO_2Cl_2 was refluxed for 1·5 hr, when 25 ml SO_2Cl_2 was added and reflux continued for 2 hr. The mixture was concentrated under reduced pressure, and the residue was washed with hot EtOAc to yield 8·6 g, 77%, of white solid, m.p. 172°, which was recrystallized from acetonitrile EtOAc. (Found: C, 37·99; H, 3·27; N, 4·92; Cl, 12·10. $C_9H_9ClNOBF_4$ requires: C, 37·88; H, 3·18; N, 4·91; Cl, 12·42); NMR (CH_3CN): 4·6 (quartet, 2), 7·3 (s, 2), 9·5 (s, 1).

Hydrolysis of benzisoxazolium salts to 2-hydroxy-N-ethyl benzamides. Hydrolyses were most conveniently carried out by heating the salt in 0·1 N HCl for several hr (min suffice for the nitro-substituted systems). Alternately, a rapid reaction occurs with $NaHCO_3$ aq, but traces of yellow impurities are formed which must be removed by extraction of the product into NaOH aq, yields of purified products lay in the range of 60–95%.

2,3 - *Dihydroxy* - N - *ethylbenzamide.* To soln of 3 - (2',3' - dihydroxybenzoyloxy) - 2 - hydroxy - N - ethylbenzamide (0·9 g; 2·8 mM) in 10 ml DMF $EtNH_2$ (0·28 g; 5·6 mM) was added. After 14 hr the solvent was evaporated at 1 mm and replaced with EtOAc. Extraction with water, drying, and evaporation yielded a black residue which was sublimed at 90°, 1 mm to give 0·66 g, 65% of product, m.p. 77–79°. The substance is very water soluble, readily oxidized, and difficult to crystallize. (Found: C, 59·70; H, 6·23; N, 7·68. $C_9H_{11}NO_3$ requires: C, 59·64; H, 6·12; N, 7·73); UV (H_2O, pH 1): 308 (3140), 247 (8760).

Acetylation of 2,3 - *dihydroxy* - N - *ethylbenzamide.* To a stirred soln of 2,3 - dihydroxy - N - ethylbenzamide (0·36 g; 2 mM) in 3 ml 1N NaOH at 0° Ac_2O (0·21 g) was added. After 1–2 min, the soln was acidified to pH 1, and the solids collected and recrystallized from EtOAc to yield 0·33 g, 73%, of ester, m.p. 134–136°. Mixture m.p. with ester prepared by reaciton of 2 - ethyl - 2 - hydroxybenzisoxazolium fluorobroate with acetate ion:

Table 6. Properties and analytical data for 2-hydroxy-N-ethylbenzamides

Substitution	m.p.	Purification		Analysis C	H	N	S or Cl
3-methoxy	114–115°	sublimation		61·43	6·61	7·29	
			$C_{10}H_{13}NO_3$ requires:	61·51	6·72	7·18	
3-methyl	71–73°	sublimation		67·05	7·35	7·76	
			$C_{10}H_{13}NO_2$ requires:	67·02	7·31	7·82	
3-hydroxy-5-nitro	227–228°	EtOH-water		47·90	4·63	12·50	
			$C_9H_{10}N_2O_5$ requires:	47·79	4·46	12·38	
3-methoxy-5-nitro	150–151°	EtOH-hexane		50·19	5·16	11·45	
			$C_{10}H_{12}N_2O_5$ requires:	50·00	5·04	11·66	
3-hydroxy-(4 or 6)-sulfonic acid	209–210°	EtOH-hexane		41·26	4·28	6·71	12·44
			$C_9H_{11}NO_6S$ requires:	41·37	4·24	5·36	12·27
3-hydroxy-(4 or 6)-chloro	153–155°	$CHCl_3$-hexane		49·98	4·63	6·71	16·60
			$C_9H_{10}ClNO_3$ requires:	50·13	4·68	6·50	16·44

Table 7. Active esters prepared from benzisoxazolium salts and carboxylic acids: N-ethylbenzamides

Substitution	m.p.	Yield (%)	Purification	$[\alpha]_D$ or $[\alpha]_{546}$	Analysis C	H	N
1. From 2-ethylbenzisoxazolium Fluoroborate:							
2-(Z-Gly-L-Phe)[4]	141–142°	93–97	EtOAC or CH_3CN	−22·5° (2·0, MeCN)	66·78	5·92	8·44
				$C_{28}H_{29}N_3O_6$ requires:	66·78	5·80	8·35
2-(Z-L-Phe)	133–134°	91	EtOAc	−55·5 (2·1, DMF)	69·97	5·90	6·36
				$C_{26}H_{26}N_2O_5$ requires:	69·93	5·87	6·28
2-(Z-L-Asn)[4]	137–139°	93	acetone-cyclohexane	−30·0° (3·1, Me_2CO)	61·24	5·72	10·25
				$C_{21}H_{23}N_3O_6$ requires:	61·01	5·61	10·17
2-(ε-Z-α-BOC-L-Lys-L-Tyr	115–116°	ca45 (for 3 steps from ester of α-Z-ε-BOCLys)	EtOAc-cyclohexane	−46·2 (2·3, MeCN)	64·20	6·84	8·02
				$C_{37}H_{46}N_4O_9$ requires:	64·33	6·71	8·11
2-(Z-(Gly-Leu-Gly)$_2$)[4]	172–173°	89	MeCN	−19·7 (4·3, DMF)	58·80	7·08	12·91
				$C_{37}H_{51}N_7O_{10}$ requires:	58·95	6·82	13·01
2. From 4,6-Dihydroxy-2-ethylbenzisoxazolium Fluoroborate							
6-hydroxy-2-(Z-Gly-L-Phe)	194–195°	85	DMF-EtOAc	+9·3	62·47	5·68	7·99
				$C_{28}H_{28}N_3O_8$ requires:	62·80	5·46	7·85
3. From 7-Methoxy-2-ethylbenzisoxazolium Fluoroborate							
2-(Z-Gly)-	81–83°	84	EtOAc-hexane		62·11	5·98	7·18
				$C_{20}H_{22}N_2O_6$ requires:	62·18	5·74	7·25
2-(Z-Gly-L-Phe)	148–150°	92	MeCN-EtOAc	−25·2 (2·0, DMF)	65·30	5·90	7·77
				$C_{29}H_{31}N_3O_7$			
			70	requires:	65·26	5·86	7·88
2-acetoxy	74·0–74·5°	EtOAc		EtOAc	60·78	6·45	5·96
				$C_{12}H_{13}NO_4$ requires:	60·73	6·38	5·91
4. From 7-methyl-2-ethylbenzisoxazolium Fluoroborate							
2-(Z-Gly)	130–131°	90	EtOAc		64·65	6·00	7·35
				$C_{20}H_{22}N_2O_5$ requires:	64·83	5·99	7·57
2-(Z-Gly-L-Phe)	125–127°	82	EtOAc	−47·8 (2·0, DMF)	67·28	6·08	8·12
				$C_{29}H_{31}N_3O_6$ requires:	67·29	6·04	8·12
5. From 5-Nitro-2-ethylbenzisoxazolium Fluoroborate							
5-nitro-2-(Z-Gly)	112–115°	33	cyclohexane		56·77	4·89	10·18
				$C_{19}H_{19}N_3O_7$ requires:	56·85	4·77	10·47
6. From 7-Hydroxy-2-ethylbenzisoxazolium Fluoroborate							
2-hydroxy-3-acetoxy	135–137°	77	EtOAc		59·37	5·83	6·29
				$C_{11}H_{13}NO_4$ requires:	59·16	5·87	6·28
2-hydroxy-3-(2′,3′-dihydroxy-benzoyl)	163–164°	163–164°	70		60·70	4·91	4·14
				$C_{16}H_{15}NO_6$ requires:	60·54	4·77	4·41
7. From 5-Nitro-7-Hydroxy-2-ethylbenzisoxazolium Fluoroborate							
2-hydroxy-3-(Z-Gly)-5-Nitro	157–158°	38	EtOAc-hexane		54·74	4·69	10·00
				$C_{19}H_{19}N_3O_8$ requires:	54·68	4·59	10·07
2-hydroxy-3-(Z-Gly-L-Phe)-5-Nitro	153–154°	30	EtOAc-hexane	−25·4 (2·0, EtOAc)	59·48	5·02	9·77
				$C_{28}H_{28}N_4O_9$ requires:	59·57	5·00	9·92

134–136°; NMR ($CDCl_3$): 1·2 (3, t), 2·3 (3, s), 3·4 (2, quintet), 6·4–7·3 (4, m).

2-Methoxy-3-hydroxy-N-ethylbenzamide. To a soln of 3 - acetoxy - 2 - hydroxy - N - ethylbenzamide (0·91 g; 4·1 mM), in 15 ml CH_2Cl_2 was added a 4-fold excess of CH_2N_2 in 100 ml ether, followed by 0·6 ml ether containing 1 drop of fluoroboric acid. The mixture was stirred at 0° for 3 hr and at 20° for 20 hr. A sticky ppt was removed by filtration and discarded, and the filtrate was evaporated. The residue was taken up in 5 ml of 0·1 M NaOH (1 hr stirring), the soln was extracted with EtOAc, acidified to pH 1, and reextracted with EtOAc. The latter extract was washed with $NaHCO_3$ aq and water, dried, and evaporated. Crystallization of the residue from $CHCl_3$ gave 0·44 g, 55%, solid, m.p. 91–92·5°, mixture m.p. with 3 - methoxy - 2 - hydroxy - N - ethylbenzamide: 66–75°. (Found: C, 61·53; H, 6·53; N, 7·13. $C_{10}H_{13}NO_3$ requires: C, 61·51; H, 6·72; N, 7·18): NMR ($CDCl_3$): 1·3 (3, t), 3·5 (2, quint), 3·9 (3, s), 6·8–7·6 (3, m), 7·7–8·3 (1, s), 8·5 (1, s).

3 - Hydroxy - N - ethylbenzamide. 3 - Acetoxybenzoyl chloride, (20 g; 0·1 mole) was dissolved in CH_2Cl_2, chilled, and treated with a soln of $EtNH_2$ (14 g, 0·3 mole) in CH_2Cl_2. The solvent was removed, the residue was taken up in 100 ml 1 N NaOH, and the resulting soln acidified to pH 1. Recrystallization from MeOH yielded 10·5 g, 64%, m.p. 158–159°. (Found: C, 65·25; H, 6·82; N, 8·43. $C_9H_{11}NO_2$ requires: C, 65·44; H, 6·71; N, 8·48)).

7 - (N - Ethylcarboxamido) - 8 - hydroxyquinoline. Ethyl 8 - hydroxyquinoline - 7 - carboxylate, prepared from 9·5 g of the acid, was added to 200 ml 33% aqueous ethylamine, and the soln was refluxed overnight. The solvent was evaporated, and the residue was taken up in EtOAc and extracted with $NaOH_3$ aq, followed by 2 × 100 ml of 1 N NaOH. The alkaline extract was washed with $CHCl_3$, acidified with HOAc, and extracted with $CHCl_3$. The latter extract was washed with H_2O, dried, and evaporated, and the residue was recrystallized from EtOAc, to give 4·5 g, 42%, m.p. 173–175°. (Found: C, 66·65; H, 5·59; N, 12·95. $C_{12}H_{12}N_2O_2$ requires: C, 66·50; H, 5·62; N, 12·97).

7 - (N - Ethylcarboxamido) - 8 - carbobenzoxyglycyloxy) - quinoline (17). A soln of carbobenzoxyglycine, (2·9 g; 14 mM) and Et_3N (1·4 g, 14 mM) in 40 ml THF was stirred and cooled to −15° as ethyl chloroformate (1·5 g, 14 mM) was added dropwise. The solvent was removed, and the residue was taken up in EtOAc, washed with $NaHCO_3$ aq and HOAc solns and water, dried (Na_2SO_4), and evaporated. Crystallization from EtOAc gave 3·0 g, 54%, m.p. 118–122°. The material decomposed readily on storage. Repeated recrystallization gave material of m.p. 126–128°. (Found C, 64·70; H, 5·26: N, 9·99. $C_{22}H_{21}N_3O_5$ requires: C, 64·86; H, 5·20; N, 10·31); IR (Nujol): 3410, 3295, 1766, 1675, 1655.

A 0·5 mM sample of this ester bearing 2-^{14}C-glycine was added to 25 ml of 2:3 v/v H_2O-DMSO soln containing 0·55 mM each of L-proline and tetramethylguanidine. After reaction was complete, isotopic dilutions revealed that 52% ZGly-L-ProOH and 0·6% of ZGlyOH had been formed. From a 2·0 mM scale experiment, a crystalline, neutral product was isolated in small yield, m.p. 280°, whose ^{14}C content implied a MW of 310, under the assumption of one glycine residue per molecule. (Found: C, 59·75; H, 4·49; N, 13·75. $C_{15}H_{13}N_3O_4$ requires: C, 60·19; H, 4·38; N, 14·04): IR (nujol): 1761, 1701, 1650. (Compare with 1760, 1705, 1650 observed for the corresponding product derived by base treatment of 4, R = ZGly.)

General procedure for the preparation of activated esters from benzisoxazolium salts and carboxylic acids. The acid is dissolved in an equivalent of 0·3N NaOH, and 0·05 to 0·07 times this volume of pyridine is added. The pH is adjusted of 4·5 to 5·0 by the addition of HCl, an equal volume of EtOAc or CH_2Cl is added, and the soln is stirred vigorously and chilled during the addition of 1·1 equivs of finely powdered benzisoxazolium salt. (With 2-ethylbenzisoxazolium fluoroborate, the addition could be rapid; with other benzisoxazolium salts, the addition was carried out in small portions over a 10–60 min interval.) When the addition is complete, the mixture is stirred for 5 min, the layers are separated, and the aqueous layer is extracted twice with EtOAc and discarded. The pooled organic phases are extracted with water containing 1 N HCl sufficient to neutralize twice the initially added pyridine, water, $NaHCO_3$ aq, and water. The solution was dried and evaporated. The residue was recrystallized from a suitable solvent.

Representative coupling reactions with benzisoxazole-derived active esters

A. *Carbobenzoxy-L-phenylalaninyl-L-valine methyl ester.* A soln of 2·40 g L-valine methyl ester hydrochloride (14·3 mM) and 1·45 g triethylamine (14·3 mM) in 15 ml DMF was treated with 6·20 g (13·9 mM) 2 - carbobenzoxy - L - phenylalaninyloxy - N - ethylsalicylamide, and the mixture was allowed to stand at room temp for 48 hr, whereupon the DMF was evaporated at 40°, 0·4 mm. The residue was dissolved in 30 ml CH_2Cl_2 and extracted with water, 10 ml 0·2 N HCl, 2 × 30 ml 0·5 N NaOH, and water, then dried and evaporated. The residue was recrystallized from EtOAc-cyclohexane to yield a first crop, 4·59 g, m.p. 113·5–114·0°, and a second crop, 0·80 g, m.p. 108–111°. Recrystallization yielded 4·95 g ester, m.p. 114·0–115·0°, 86·5%, $[\alpha]^{546} - 19·7°$ (2·2, MeOH). (Found: C, 66·89; H, 6·95; N, 7·01. $C_{23}H_{28}N_2O_5$ requires: C, 66·97; H, 6·84; N, 6·79).

B. *t - Butoxycarbonyl - L - glutaminyl - L - tryptophane methyl ester.* The ester 2 - (BOC - L - Gln) - N - ethylsalicylamide was prepared from BOC-L-GlnOH and 2-ethylbenzisoxazolium fluoroborate, m.p. 145·0–146·0°; (Found: C, 57·79; H, 6·94; N, 10·88; $C_{19}H_{27}N_3O_6$ requires: C, 58·00; H, 6·92; N, 10·68%).

This ester (3·43 g; 87 mM) was added to a soln of L-tryptophane methyl ester hydrochloride (2·35 g; 9·2 mM) and triethylamine (0·92 g; 9·2 mM) in 12 ml DMF. After 50 hr at 20°, the DMF was evaporated ($T < 40°$), the residue was taken up in 100 ml EtOAc which was then extracted with 3 × 20 ml 1% citric acid and 2 × 20 ml 0·5 N NaOH. Drying and evaporation, followed by washing with EtOAc yielded 3·42 g ester, 88%, m.p. 168·0–169·0° dec. Recrystallization from acetonitrile EtOAc yielded material, m.p. 171–172° dec. $[\alpha]_D^5 = 10·8$ (2·0, DMF). (Found: C, 59·26; H, 6·94; N, 12·38. $C_{22}H_{30}N_4O_6$ requires: C, 59·19; H, 6·77; N, 12·55%).

Acknowledgements—Financial support from National Institutes of Health Grant GM 13453 is gratefully acknowledged.

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