cyclobutene **4**. We are presently exploring the nature and generality of substituent effects in these systems.

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1-Hydroxybenzotriazole as a Racemization-Suppressing Reagent for the Incorporation of *im*-Benzyl-L-histidine into Peptides

Sir:

Racemization during the synthesis of polypeptides can be a serious problem because a high degree of steric homogeneity is usually necessary for biological or physical studies. The detection of small amounts of diastereoisomeric contaminants and their removal from synthetic polypeptide preparations is often difficult, so synthetic procedures which minimize the risk of racemization are commonly used. The most widely used procedure, particularly for the synthesis of relatively short polypeptide chains, involves the stepwise synthesis from the carboxyl end using urethane-protected amino acids.[1] The popularity of this method rests largely on the belief that racemization does not occur. Recently, however, it was shown that considerable racemization of *tert*-butyloxycarbonyl-*im*-benzyl-L-histidine [Boc-His(Bzl)] had occurred under the somewhat more demanding conditions of solid-phase peptide synthesis.[2,3] In this case, removal of the contaminating isomers was not possible. Subsequent studies utilizing a model system based upon the separation and quantitation of diastereoisomers of *im*-benzylhistidylglutamic acid [His(Bzl)-Glu] on an amino acid analyzer demonstrated that Boc-His(Bzl) racemizes under a variety of conditions, including standard solution reactions.[4] It was found that the presence of 1 equiv of *N*-hydroxysuccinimide virtually eliminated racemization in the dicyclohexylcarbodiimide-mediated coupling of Boc-His(Bzl) with Glu(OBzl)-O-polymer. However, in agreement with the findings of Kaiser, *et al.*,[5] the yield in this reaction was unacceptably low (70%). The presence of 30% β-alanine in the peptide hydrolysates suggests that the low yield was due to competitive acylation of the amino function by succinimidoxycarbonyl-β-alanine-*N*-hydroxysuccinimide ester, a known product of the reaction between dicyclohexylcarbodiimide and *N*-hydroxysuccinimide.[6,7]

Recently, König and Geiger[8] showed that 1-hydroxybenzotriazole was as effective as *N*-hydroxysuccinimide in suppressing racemization in dicyclohexylcarbodiimide-mediated couplings where oxazolone formation is possible. Since a side reaction comparable to the one between dicyclohexylcarbodiimide and *N*-hydroxysuccinimide seemed unlikely for 1-hydroxybenzotriazole, it seemed worthwhile to investigate the utility of this reagent in the synthesis of His(Bzl) peptides. Boc-His(Bzl), 34.5 mg (0.1 mmol), Glu(OBzl)-OBzl·Tos, 50 mg (0.1 mmol), Bu_3N, 0.024 ml (0.1 mmol), and 1-hydroxybenzotriazole, 13.5 mg (0.1 mmol), were dissolved in 1 ml of CH_2Cl_2.[9] To this solution was added dicyclohexylcarbodiimide, 20.5 mg (0.1 mmol); the solution was kept at 23° overnight. The precipitated dicyclohexylurea was removed by filtration and the filtrate evaporated at 23° under vacuum. The residue was dissolved in 5 ml of CF_3COOH, and HBr was bubbled through the solution for 2 hr. The solution was then evaporated under vacuum at 23° and the residue was dissolved in 30 ml of 0.2 *N* sodium citrate, pH 2.2. A 0.5-ml aliquot was chromatographed on a 0.9 × 18 cm column of Beckman PA-35 resin using 0.35 *N* sodium citrate, pH 5.26, for elution at 70 ml/hr. Under these conditions D-His(Bzl)-L-Glu elutes in 44 min and L-His(Bzl)-L-Glu in 55 min. Less than 0.1%[10] D-His(Bzl)-L-Glu was found in this case, compared with 1.8% when 1-hydroxybenzotriazole was omitted.

For the solid-phase test, 330 mg (0.04 mmol) of Glu(OBzl)-O-polymer was agitated gently with a solution of 55 mg (0.16 mmol) of Boc-His(Bzl) and 22 mg (0.16 mmol) of 1-hydroxybenzotriazole in 1.5 ml of CH_2Cl_2. After 10 min, 33 mg (0.16 mmol) of dicyclohexylcarbodiimide was added, and agitation continued overnight. The polymer was collected on a sintered filter, washed with CH_2Cl_2 (three 3-ml portions), 50% CF_3COOH in CH_2Cl_2 (three 3-ml portions), and CF_3COOH (3 ml), then suspended in 5 ml of CF_3COOH, and HBr was bubbled through the suspension for 2 hr. After filtration and evaporation, the crude reaction product was submitted to chromatographic analysis as before. Less than 0.3% D-His(Bzl)-L-Glu was found, compared with 11% in the absence of 1-hydroxybenzotriazole.

In order to evaluate the yield achievable during solid-phase synthesis, Pro-Phe-O-polymer was acylated with Boc-His(Bzl), 1-hydroxybenzotriazole, and dicyclohexylcarbodiimide as described above. A portion of the crude product obtained by HBr cleavage was hydrolyzed in constant-boiling HCl at 110° under N_2 for 24 hr. An aliquot of the hydrolysate subjected to amino acid analysis had the expected amino acids in equimolar amounts: His(Bzl), 1.03;[11] Pro, 0.98; Phe, 1.00. An L-amino acid oxidase digest[2] of the hydrolysate had His(Bzl), 0.00; Pro, 1.00; Phe, 0.00 (after correcting for the amount of racemization occurring during acid hydrolysis), providing independent proof that racemization had been negligible. The equality of Pro and Phe in the acid hydrolysate shows that no side reactions had occurred which could have resulted in irreversible modification of Pro, thus validating the use of amino acid ratios to evaluate cou-

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(10) The sensitivity limit of the test.
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pling yields. When a portion of the unhydrolyzed peptide solution was chromatographed on the 0.9 × 18 cm PA-35 column (eluted with 0.38 *N* sodium citrate, pH 7.00, at 70 ml/hr), less than 5% free His(Bzl) was present, indicating that removal of noncovalently bound His(Bzl) prior to cleavage was almost complete; thus the His(Bzl) in the hydrolysates came primarily from the peptide. High-voltage paper electrophoresis at pH 1.85 showed a single ninhydrin and chlorine + spot.[12]

These results indicate essentially quantitative reaction between Boc-His(Bzl) and Pro-Phe-O-polymer under the conditions used. No attempts have yet been made to refine the reaction conditions to determine if reaction time or molar excesses of reagents could be reduced without sacrificing yields or optical purity. The tripeptide has been synthesized on a larger (0.5 mmol) scale as a chromatographically homogeneous product in a quantitative yield. As with the analytical scale experiment, the crude product had His (Bzl), Pro, and Phe in equimolar amounts; however, after hydrogenation (48 hr at 30 psi in 10% AcOH in the presence of an equal weight of 10% Pd/C) the Phe content had dropped to 92% and ~8% hexahydrophenylalanine was present, despite the fact that 1–2% His(Bzl) remained. This illustrates an undesirable feature in the use of His(Bzl) in peptide synthesis, the occasional difficulty in selective removal of the benzyl group.

The results described show that 1-hydroxybenzotriazole is effective in reducing racemization during solid-phase peptide synthesis with Boc-His(Bzl) to acceptably low levels without impairing coupling efficiency. It is hoped that this technique will also be useful with histidine analogs, where the use of basicity-suppressing protecting groups for racemization control[4] is not possible.

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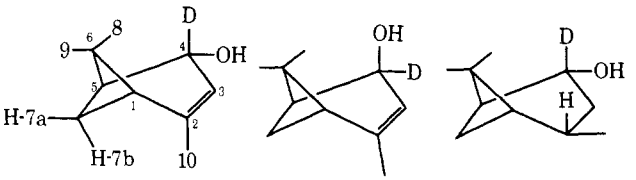
Isotope Effects in Nuclear Magnetic Resonance Spectra Modified by Rare-Earth Shift Reagents

Sir:

Since the original discovery of a rare-earth nuclear magnetic resonance shift reagent,[1] considerable exploration of the field has occurred[2] and new reagents have been found.[3] Although the general theory and utility of the method are well into their formative stages, we wish to report an unusual and novel isotope effect which is not only unexpected, but also discloses new aspects of the nature of the shift reagent-substrate interaction.

In a series of experiments designed to test for the occurrence of [1,3] sigmatropic shifts during catalytic hydrogenation,[4,5] we had occasion to label the verbenols at the alcohol carbon. After a preliminary hydrogenation of a mixture of light and heavy *trans*-verbenol,



1 2 3

4-deuterio-*trans*-verbenol | 4-deuterio-*cis*-verbenol | 4-deuterioverbanol

the verbanol was purified by gas–liquid chromatography and examined by nmr using Hinckley's reagent (the dipyridine adduct of trisdipivalomethanatoeuropium(III), $Eu(DPM)_3 \cdot 2py$).[1] Concomitant with the expected shifts, a doubling of all hydrogen peaks occurred. The origin of the extra set of peaks was readily apparent when the downfield set increased upon addition of a pure sample of 4-deuterioverbanol to the mixture. Examination of a variety of alcohols and one aldehyde (2-methylbenzaldehyde-d_1, **4**) shows that the effect is quite general (Table I).

Table I. Isotope Effects on Nmr Shifts Induced by $Eu(DPM)_3 \cdot 2py$

Labeled compd	Resonance obsd	Chemical shift,[a] Hz	Shift changes,[b] Hz	% difference[c]
1	Methyl-8	105	−170	3.1
	Methyl-9	132	−67	3.3
	Olefinic	530	−206	2.9
	H-7a	239	−69	2.7
	H-7b	126	−86	2.6
2	Methyl-8	86	−96	1.6
	Olefinic	530	−361	1.4
	H-7b	130	−408	1.7
4	Methyl	265	−38	4.3

[a] At 100 MHz. Initial chemical shift is same for both labeled and unlabeled compounds. [b] At 100 MHz. Induced changes in shifts are reported for unlabeled material; only enough shift reagent was added in each case to a 50/50 mixture to allow accurate measurement of shift differences between labeled and unlabeled molecules. Per cent difference appears to be independent of shift reagent concentration. [c] (Induced shift of labeled compound − induced shift of unlabeled compound)100/induced shift of unlabeled compound.

This finding indicates a greater association constant between the deuterium-substituted compound and the metal complex than between the light compound and metal complex. Clearly, in addition to simple metal–oxygen association, there exists a contribution to bonding which is enhanced by the presence of a deuterium atom in place of the hydrogen atom geminal to the hydroxyl group.

Variations in the concentration of alcohol, metal complex, and pyridine produced no change in the observed per cent difference for 2-butanol and a number of other alcohols studied. These results suggest that the differences in effective metal chelate–substrate association constants for the light and heavy molecules studied are due principally to differences in metal chelate–substrate complex stability.

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