$C_2B_9H_{11}$. Nevertheless, a study of the nmr spectrum of $B_{10}H_{10}CH^-$ at higher temperatures might be interesting.

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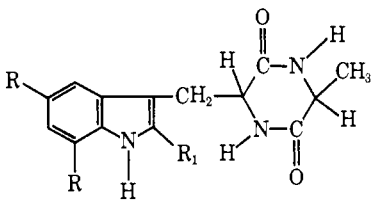
## Monoisoprenylated *cyclo*-L-Alanyl-L-tryptophanyl. A Biosynthetic Precursor of Echinulin

*Sir:*

Biosynthetic studies in *Aspergillus amstelodami* indicate that mevalonic acid[1] and *cyclo*-L-alanyl-L-tryptophanyl[2] are *in vivo* precursors of echinulin (I). Partially isoprenylated peptide intermediates have not been isolated from the fungus or been shown to be *in vivo* precursors of echinulin. Recently, however, a partially purified enzyme from this fungus has been described[3] which transfers the isoprene unit from 3-methyl-2-butenyl 1-pyrophosphate to *cyclo*-L-alanyl-L-tryptophanyl (III) forming monoisoprenylated *cyclo*-L-alanyl-L-tryptophanyl (MICAT), tentatively identified as *cyclo*-L-alanyl-2-(1,1-dimethylallyl)-L-tryptophanyl (II). This



I, R = $-CH_2CH{=}C(CH_3)_2$; $R_1 = -C(CH_3)_2CH{=}CH_2$
II, R = H; $R_1 = -C(CH_3)_2CH{=}CH_2$
III, R = $R_1$ = H

paper describes *in vivo* studies which establish MICAT as a precursor of echinulin.

*cyclo*-L-Alanyl-L-tryptophanyl, *cyclo*-L-alanyl-L-[3-$^{14}$C]tryptophanyl, and [1-$^3$H]3-methyl-2-butenyl 1-pyrophosphate were prepared as previously described.[3] MICAT, singly labeled with tritium in the isoprene moiety and doubly labeled with tritium in the isoprene moiety and $^{14}$C in the 3 position of the tryptophanyl moiety, were prepared enzymically using the above reagents and a partially purified enzyme from *A. amstelodami*, by a slight modification of the previously described methods.[3] The chromatographic mobilities and ultraviolet spectra of the isolated radioactive products were the same as those previously described for MICAT.

The utilization of $^3$H-MICAT and $^3$H,$^{14}$C-MICAT as precursors of echinulin was studied in growing surface cultures of *A. amstelodami* (ATCC 10065). Culture flasks containing 50 ml of Czapek-Dox broth supplemented with sucrose (30%) were inoculated with fungus and incubated at 30°. In two experiments, 2- to 3-day-old cultures were fed $^3$H-MICAT dissolved in 0.25 ml of dimethyl sulfoxide (251,400 dpm, experiment 1; and 282,000 dpm, experiment 2), and then permitted to continue growing for 4 more days. In a third experiment, a 4-day-old culture was similarly fed $^3$H,-$^{14}$C-MICAT (334,000 dpm, $^3$H; and 12,500 dpm, $^{14}$C) and permitted to continue growing for 3 more days. The fungal mats were harvested and dried, and the lipid-soluble metabolites were extracted with $CHCl_3$ as previously described.[4] The $CHCl_3$ extracts contained approximately 20% of the total radioactivity fed to the fungus. Most of the remaining radioactivity was shown to be present in the culture medium.

In each case the $CHCl_3$ extracts were concentrated and the metabolites chromatographed as previously described[4] on 10-g silica gel columns, using 250 ml each of benzene–ethyl acetate (8:2, v/v) and benzene–butanol (95:5, v/v) as eluents. The metabolites eluted in several ultraviolet absorbing peaks with echinulin emerging from the column in the benzene–butanol solvent as previously described.[4] Echinulin was identified by its ultraviolet spectrum and $R_f$ values in several thin-layer chromatographic systems. Radioactivity was observed in the echinulin fraction in each case and represented 14, 11, and 5% of the total radioactivity fed to the fungus in experiments 1, 2, and 3, respectively.

Aliquots from the pooled chromatographic fractions containing echinulin (experiment 1) were subjected to thin-layer silica gel chromatography in three solvent systems, benzene–ethyl acetate (8:2, v/v), benzene–butanol (8:2, v/v), and benzene–ethanol (8:2, v/v), and gave $R_f$'s of 0.00, 0.85, and 0.82, respectively. In each case, the only component observed on the fluorescent sheets chromatographed with an $R_f$ value identical with that of authentic echinulin. Furthermore, analysis of the chromatographic sheets for radioactivity indicated in each solvent system that essentially all of the radioactivity cochromatographed with echinulin. *cyclo*-L-Alanyl-L-tryptophanyl and the MICAT in benzene–butanol (8:2, v/v) gave $R_f$'s of 0.11 and 0.45, respectively, indicating that the fungal product chromatographs quite differently than these compounds.

In other control experiments, approximately $5 \times 10^4$ dpm of the $^3$H-MICAT was mixed with either 33 mg of echinulin or a nonradioactive $CHCl_3$ extract of a fungal mat grown under the same conditions as those used in the feeding experiments. Silica gel column chromatography of these mixtures demonstrated no radioactivity in the isolated echinulin fractions. The $^3$H-MICAT was removed from these columns by subsequent elution with ethanol.

Furthermore, a crude $^3$H-labeled echinulin fraction (8500 dpm), prepared by differential solvent extraction[5] of a dried $CHCl_3$ extract from a culture fed $^3$H-MICAT, was mixed with 30 mg of authentic echinulin and subjected to repeated recrystallizations from ethanol. Constant specific activity was obtained after the fourth recrystallization.

Experiments with doubly labeled MICAT were car-

(1) A. J. Birch, G. E. Blance, S. David, and H. Smith, *J. Chem. Soc.*, 3128 (1961).
(2) G. P. Slater, J. C. MacDonald, and R. Nakashima, *Biochemistry*, **9**, 2886 (1970).
(3) C. M. Allen, Jr., *Biochemistry*, **11**, 2154 (1972).
(4) C. M. Allen, Jr., *Can. J. Microbiol.*, **18**, 1275 (1972).
(5) J. C. MacDonald and G. P. Slater, *Can. J. Microbiol.*, **12**, 455 (1966).

ried out to eliminate the possibility that radioactivity found in echinulin was due to degradation of the $^3$H-MICAT to small tritiated compounds which would serve as precursors of echinulin. Table I compares the

**Table I.** Incorporation of $^3$H,$^{14}$C-MICAT into Echinulin

| | Radioactivity | | | Moles isoprene[a]/mole cyclic dipeptide |
|---|---|---|---|---|
| | $^{14}$C $\mu$Ci $\times 10^{+5}$ | $^3$H $\mu$Ci $\times 10^{+3}$ | ($\mu$Ci $^{14}$C/$\mu$Ci $^3$H) $\times 10^{-2}$ | |
| $^3$H,$^{14}$C-MICAT | 5.80 | 1.53 | 3.79 | 1.16 |
| Echinulin fraction from silica gel chromatography | 2.59 | 0.68 | 3.81 | 1.17 |
| Recrystallized echinulin | 2.91 | 0.76 | 3.83 | 1.17 |

[a] Specific activities were determined as previously described.[3] Ratios are accurate to ±10%.

incorporation of $^{14}$C and $^3$H from the doubly labeled MICAT into samples of echinulin isolated in experiment 3. The results indicate the same ratio of $^{14}$C to $^3$H in MICAT as observed in echinulin, indicating that echinulin biosynthesis from metabolic breakdown products of MICAT is highly improbable.

It is apparent from these experiments that monoisoprenylated *cyclo*-L-alanyl-L-tryptophanyl is a good biosynthetic precursor of echinulin and is a likely intermediate on the natural metabolic pathway.

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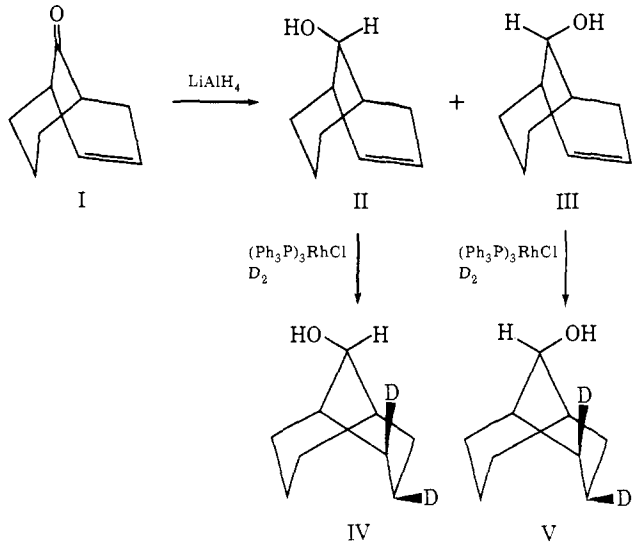
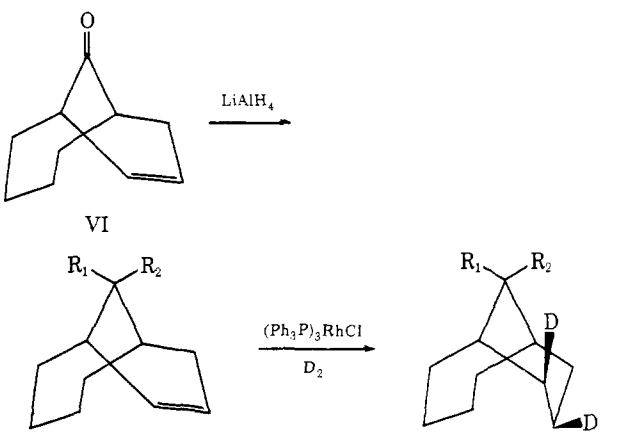
## An Unequivocal Stereochemical Assignment by Mass Spectrometry

*Sir:*

There have been many attempts to utilize mass spectrometry for stereochemical assignments in cyclic systems,[1] and considerable effort also has been devoted to analyzing those factors which determine the specificity of mass spectrometric loss of water from cyclic alcohols.[2] Although it seems reasonably well accepted that such specificity as exists in this elimination process in six-membered rings (1:4 cis > 1:3 loss) is determined by the spatial relationships of the hydroxyl group with neighboring hydrogen atoms in the molecular ion (proximity effects), attempts to use this acquired knowledge for stereochemical assignments have not proved to be particularly convincing.[1] Green[2b] has predicted that "deuterium labeling studies of electron impact induced elimination reactions are the likely road to gain the so far unrealized but expected potential for mass spectrometry in stereochemical studies." We wish to report the use of precisely this approach to determine the configuration of the hydroxyl substituent on the bridging carbon atom in 2-bicyclo[4.3.1]decen-10-ol.




VIIa, $R_1$ = OH; $R_2$ = H
b, $R_1$ = H; $R_2$ = OH

VIIIa, $R_1$ = OH; $R_2$ = H
b, $R_1$ = H; $R_2$ = OH
c, $R_1R_2$ = O

While undertaking a general study into the specificity of mass spectrometric water loss in isomeric bicyclo[3.3.1]nonanols,[3] we had occasion to prepare two cis deuterated 9-ols IV and V. These were readily available *via* lithium aluminum hydride reduction of 2-bicyclo[3.3.1]nonen-9-one (I) followed by homogeneous catalytic reduction of the double bond using tris(triphenylphosphine)rhodium chloride and deuterium gas in dry benzene solution. The anti (II) and syn (III) compounds were separated by preparative gas chromatography. In order to substantiate that specific cis deuteration had occurred at the exo face in II and III, the pmr spectra of IV and V were analyzed using the Eu(FOD)$_3$ shift reagent to separate the proton signals.[4] From the relative magnitudes of the proton shifts, the reduction in peak integration values, the splitting patterns, and the effect of the deuterium substitution on the signal shapes of adjacent protons, it was confirmed that these assignments were correct. Be-

(1) For a general review, see S. Meyerson and A. W. Weitkamp, *Org. Mass Spectrom.*, 1, 659 (1968).

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(3) J. K. MacLeod, M. R. Vegar, and R. J. Wells, *Recent Develop. Mass Spectrosc., Proc. Int. Conf. Mass Spectrosc.*, 1197 (1970).

(4) A similar study on *exo*-3-bicyclo[3.3.1]nonanol has been reported: M. R. Vegar and R. J. Wells, *Tetrahedron Lett.*, 2847 (1971).