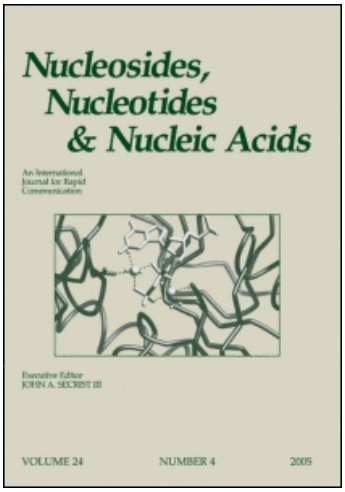

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Hartgers, W. A. , de Boer, R. F. , Wanner, M. J. and Koomen, G. J.(1992) 'A Potential Suicide Inhibitor of Adenylosuccinate Lyase', Nucleosides, Nucleotides and Nucleic Acids, 11: 7, 1325 — 1332
To link to this Article: DOI: 10.1080/07328319208021176
URL: http://dx.doi.org/10.1080/07328319208021176

NUCLEOSIDES & NUCLEOTIDES, 11(7), 1325-1332 (1992)

A POTENTIAL SUICIDE INHIBITOR OF ADENYLOSUCCINATE LYASE‡

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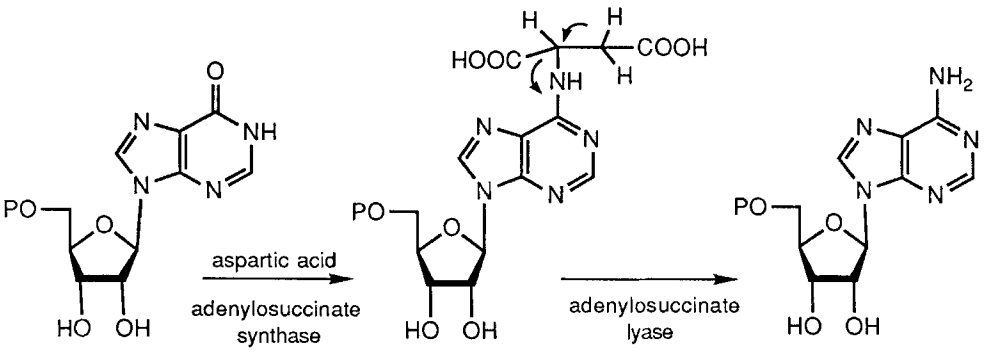
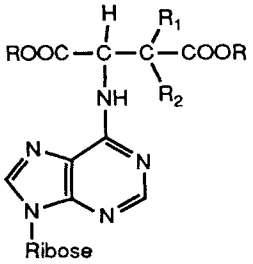
ABSTRACT Synthesis and properties of **4**, a potential suicide inhibitor of adenylosuccinate lyase, capable of producing a reactive ketene (**5**) within the active site, are described.

Adenylosuccinate lyase is an essential enzyme catalysing two steps of the biosynthesis of adenosine nucleotides. In the conversion IMP → AMP it facilitates the elimination of fumaric acid from adenylosuccinate[1]:



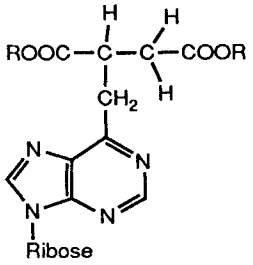
We have been engaged in the synthesis of adenylosuccinate analogues of different types. In these analogues, the elimination of fumaric acid was inhibited by either substituting the protons involved by a methyl group or a fluorine atom (**1**)[2], or by replacing the 6-amino group of adenine by a methylene function (**2**)[3]. The objective of the latter isosteric modification was to decrease the leaving group properties of the heterocyclic system.

‡ Dedicated to the memory of Prof. Tohru Ueda



R_1, R_2 = H, F, CH_3
R = C_2H_5, CH_2Ph

1



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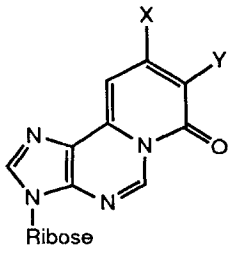


X, Y = H, $COOCH_3$

3

Our interest in the preparation and study of inhibitors of this enzyme was recently stimulated again by the following developments:

1. The activity of the enzyme was determined by Reed [4] in human tissues of different nature. It was found that the enzyme can be used as a malignancy marker, showing significantly higher activity in human mammacarcinoma than in benign breast tumors. Based on this result, we reasoned that effective inhibition of the enzyme thus could lead to a selective anticancer effect, since the malignant cells might be more sensitive to this inhibition.
2. The enzyme from yeast in purified form recently became commercially available[5], which facilitates the fundamental *in vitro* inhibition studies. There is no indication, that the enzyme from mammalian sources is fundamentally different in the region of the active site.
3. Methods for determining enzyme activity are well documented, both on the isolated enzyme as well as with homogenized tissue[4].

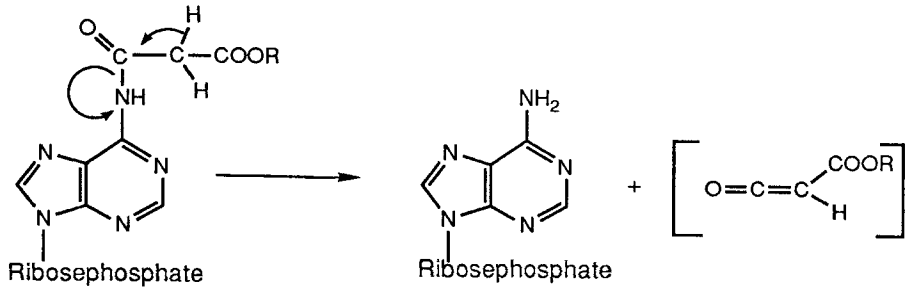
For the rational design of a nucleoside analogue with potential antitumor activity we envisaged the following procedure for synthesis and biological studies in systems of increasing complexity:

1. Synthesis of analogues as their 5' phosphate for *in vitro* studies on the isolated enzyme.
2. In case the analogue showed activity as an inhibitor of adenylosuccinate lyase, synthesis of the non-phosphorylated derivatives for studying cytostatic effects on cellgrowth *in vitro*.
3. Anticancer studies of the non-phosphorylated derivatives in a suitable murine tumour model.

In earlier work we found that carba analogues (**2**) of adenylosuccinate easily cyclized (especially under basic conditions) to pyrido-purine systems of general structure **3** with highly fluorescent properties in the visible region.[6] In order to prepare more effective inhibitors, which could be expected to be stable under biological conditions, we directed our at-

tention to the preparation of inhibitors of the suicide type. It is not simple to devise a molecule with potential suicide properties without major structural modifications. Since adenylosuccinate lyase catalyzes reactions with rather different substrates i.e. adenylosuccinate and 5-aminoimidazole-4-N-succinimidocarboxamide ribotide, we assumed that it would not exhibit high substrate specificity and would probably accept different types of substrate analogues within its active site. On the other hand, Porter reported that the corresponding nitro analogue, prepared biosynthetically from IMP and nitroalanine, was not accepted as a substrate by adenylosuccinate lyase[7]. In this case however, this may be the result of rather different charge distributions within the nitro group.

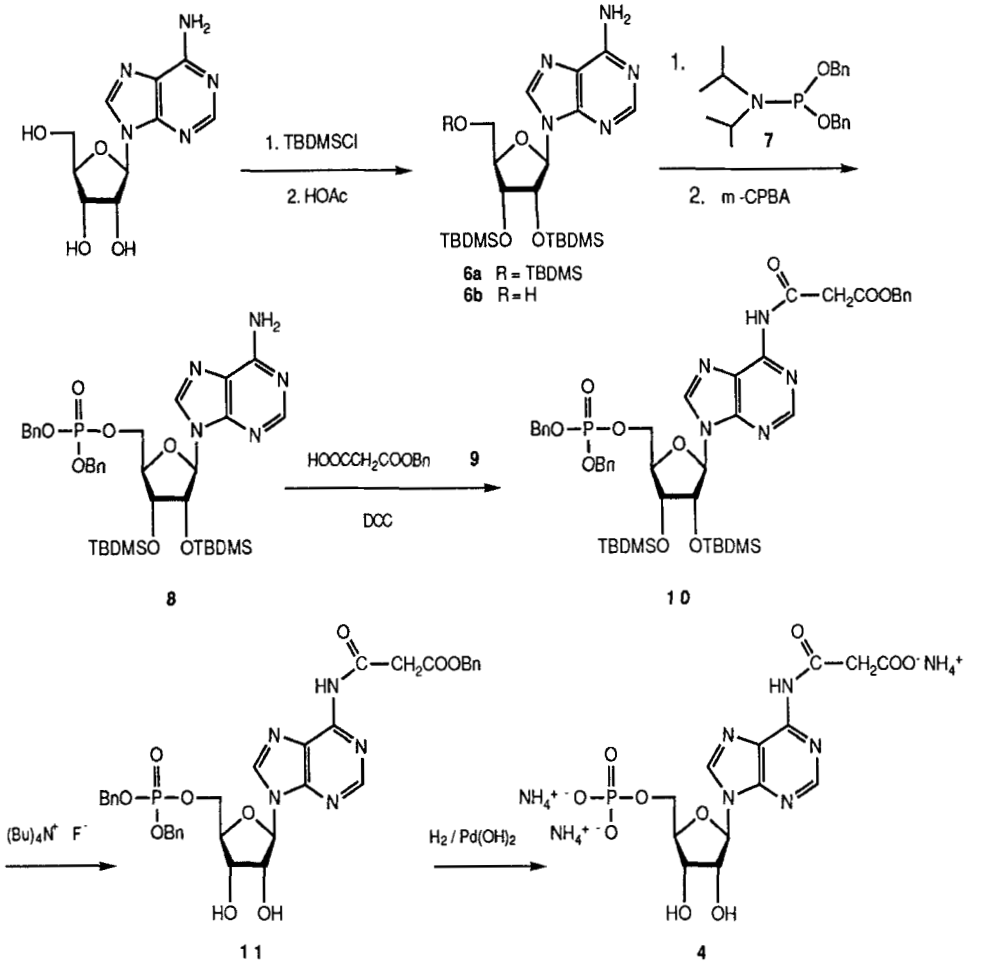
In first instance we decided to prepare derivative **4**, which might produce a very reactive ketene within the active site:



4 **5**

During synthetic experiments it became apparent, that the amide bond in **4** was hydrolysed even under very mild conditions, due to the good leaving group properties of the adenine ring. This could be the result of the elimination reaction depicted above, which needs to take place after the analogue has entered the active site of the enzyme. In every case application of acyl groups for protection of the ribose hydroxyl functions was impossible since under the conditions of hydrolysis or transesterification the synthetic products would decompose into the starting adenosine systems. This also restricted the use of a variety of carboxylic esters in the malonyl side chain. Moreover, the free carboxylic acid of **4**, carrying a β carbonyl group is very prone to decarboxylation, leading to N-acetyl adenosine derivatives. These observations also influenced the choice of the protection of the phosphate during the synthesis. Removal of the protective groups on phosphorus also should be possible under conditions which would leave the amide bond intact.

The synthesis of **4** was achieved according to the following sequence of reactions:



All hydroxyl groups in adenosine were protected in one step via a reaction with 3 eq. *t*-butyldimethylsilyl chloride in DMF using imidazole as a base[8].

Treatment of the trisilylated adenosine with 80 % acetic acid at 100° C selectively removed the 5' protection, leading to **6b** as a crystalline solid (mp. 246-250 °C). The adenosine derivative **6b** was phosphorylated in 90 % yield using N,N-diisopropyl dibenzyl phosphoramidite **7**[9] with tetrazole, followed by an oxidation to the protected phosphate with m-chloroperbenzoic acid[9]. The presence of the phosphate group was established by spectral data, including the long range coupling of the 5' methylene protons with the phosphorus nucleus. In the ^{1}H-NMR spectrum, this was visible in the more complex pattern of the two 5' methylene protons, which in nucleosides appear in D_2O as a neat ABX system. Since, however, in the spectrum the 5' methylene group signal was overlapped with 4' and 3' signals, details of the coupling constants could not be extracted from the ^{1}H NMR spectra. In ^{13}C- spectrum of the product however, the splitting of C-5' and C-4' into doublets was clearly visible. (J= 4.0 and 8.8 Hz resp)[10](see experimental part).

Mono benzyl malonate **9** (mp. 35-37 °C) was obtained via a reaction of Meldrum's acid (2,2-dimethyl-1,3-dioxane-4,6-dione) with benzyl alcohol according to the literature[11].

The coupling of **8** with **9** was carried out in pyridine using DCC as a coupling reagent (yield 69%). The remaining two silyl protecting groups were removed in 87 % yield with tetrabutyl ammonium fluoride. In the last step all protecting benzyl groups were removed by catalytic reduction using Pearlman's catalyst ($Pd(OH)_2$) in ethanol. To avoid decomposition of the product this reaction was carried out in the presence of ammonium bicarbonate buffer at pH=7-8.

The structures of intermediates and of product **4** were established by ^{13}C-NMR, ^1H-NMR and mass spectroscopy. It was interesting to note that in D_2O exchange of the protons of the malonyl methylene group was observed. In d_6-DMSO however the methylene group was clearly visible as a sharp singlet at $\delta = 3.2$. Although this methylene carbon was not visible in the ^{13}C spectrum (D_2O), due to the proton exchange, the two carbonyl signals established the structure.

The activity of **4** as an enzyme inhibitor was determined on adenylosuccinate lyase from yeast. (Sigma). A double reciprocal (Lineweaver-Burk) plot of 1/v vs 1/S showed non competitive inhibition ($K_i = 65\,\mu M$, $K_m = 3.2\,\mu M$). Although generally this is an indication of inhibition outside the active site, in the case of suicide inhibition competition of inhibitor and substrate for the enzyme cannot be expected, due to the irreversible character of the interaction.

The activity found on the enzyme stimulates us to study the cytostatic effect of **4** on *in vivo* mouse leukemia. In view of the membrane passages this requires the preparation of the non-phosphorylated analogue of **4**. The synthesis of this compound is currently underway.

Experimental section

All melting points are uncorrected. IR spectra were recorded on a Perkin Elmer 1310 spectrophotometer. The absorptions are given in cm^{-1}. NMR spectra were run on Bruker WM 250 and AC 200 instruments. Unless stated otherwise, IR and NMR spectra were taken in $CHCl_3$ and $CDCl_3$, respectively. Fast Atom Bombardment (FAB) mass spectrometry was carried out using a V.G. Micromass ZAB-HFqQ mass spectrometer, coupled to a V.G. 11/250 data system. Flash chromatography was performed on silica gel 60 (230 - 400 mesh). Thin-layer chromatography was carried out with F 254 plates.

2',3',5'-tri-(*t*-butyldimethylsilyl)adenosine (6a)
To a solution of 2.70 g (10.1 mmol) adenosine and 5.61 g (82.5 mmol) imidazole in 20 ml dry DMF was added 6.45 g (42.8 mmol) TBDMSCl under a N_2-atmosphere. After 18 h. stirring at room temperature, the reaction mixture was taken up in 50 ml ether and washed with 20 ml water. After drying ($MgSO_4$) and concentration the residue was chromatographed over

silica (EtOAc/PE : 3/1). R_f: 0.67 (EtOAc) 0.13 (EtOAc/PE 1:3). Yield: 5.37 g (8.80 mmol, 87 %) of a white foam, m.p. 142-144 °C.
^{1}H-NMR : - 0.23 (s, 3H); -0.05 (s, 3H); 0.10, 0.13 and 0.14 (3xs, 12H); 0.79, 0.93 and 0.95 (3xs, 3x9H); 3.78 (dd, J = 2.7 and 11.3 Hz, $H_{5'}$); 4.03 (dd, J = 4.0 and 11.2 Hz, $H_{5'}$); 4.12 (m, $H_{4'}$); 4.31 (dd, J = 4.0 and 3.7 Hz, $H_{3'}$); 4.67 (dd, J = 4.7 and 4.9 Hz, $H_{2'}$); 5.71 (bs, NH_2); 6.03 (d, J = 5.2 Hz, $H_{1'}$); 8.17 and 8.34 (2xs, H_2 and H_8).
IR: 3410 (s), 1625 (s), 1585 (s), 1465 (m).
Acc mass: Calc. for $C_{28}H_{55}N_5O_4Si_3$ + H : 610.36416; found $[M + H]^+$: 610.36456.

2',3'-di-(*t*-butyldimethylsilyl)adenosine (6b)
2.4 g of compound **6a** (3.9 mmol) is stirred for 4 hours in 5 ml 80% AcOH at 100 °C. After concentration the residue is evaporated twice with toluene. The residue is stirred in ether, the precipitate is filtered off and recrystallized from EtOAc/ether. This yielded 1.9 g of white crystals (3.8 mmol, 98 %). M.p. 246-250 °C. R_f: 0.38 (EtOAc).
^{1}H-NMR : - 0.63 (s, 3H); -0.15 (s, 3H); 0.10 and 0.11 (2xs, 6H); 0.73 and 0.93 (2xs, 2x9H); 3.96-3.66 (2x dd, J = 11.7 and 13.1 Hz, $H_{5'}$); 4.15 (m, $H_{4'}$); 4.31 (d, J = 4.4 Hz, $H_{3'}$); 5.02 (dd, J = 4.5 and 7.9 Hz, $H_{2'}$); 5.77 (d, J = 7.9 Hz, $H_{1'}$); 6.25 (bs,NH_2); 6.73 (d, J = 4.5,OH); 7.83 and 8.31 (2xs, H_2 and H_8).
IR : 3410 (s), 1625 (s), 1590 (m), 1465 (m).
Acc mass: Calc. for $C_{22}H_{41}N_5O_4Si_2$ + H :496.27768; found $[M + H]^+$: 496.27809.

Dibenzyl ester of 2', 3' di(*t*-butyldimethylsilyl)-adenosine-5' phosphate (8)
Under a N_2-atmosphere 0.91 g N,N-diisopropyl dibenzyl phosphoramidite (**7**, 2.6 mmol) was added to a solution of 1.00 g **6b** (2.0 mmol) and 0.28 g 1H-tetrazole (4 mmol) in 10 ml dry CH_2Cl_2. After two hours the reaction mixture was cooled to -40 °C and 0.69 g m-CPBA (3.40 mmol) was added, the mixture stirred for 45 min. at 0 °C and 15 min. at room temperature. The reaction mixture was taken up in 50 ml CH_2Cl_2 and washed with 2x 20 ml 10% $NaHSO_3$ solution, 2x 15 ml saturated $NaHCO_3$ solution, 15 ml water and 15 ml brine and dried over Na_2SO_4. This yielded after evaporation and flash chromatography 1.77 g of white crystals (2.3 mmol, 90 %). M.p. 91-94 °C. R_f: 0.46 (EtOAc).
^{1}H-NMR: - 0.24 (s, 3H); -0.05 (s, 3H); 0.07 (2xs, 6H); 0.79 and 0.90 (2xs, 2x9H); 4.34-4.14 (m, $H_{3'}$, $H_{4'}$, $H_{5'}$); 4.86 (dd, J = 4.6 Hz, $H_{2'}$); 5.03 (m, 2x CH_2Ph); 5.79 (bs,NH_2); 5.91 (d, J = 4.8 Hz, $H_{1'}$); 7.32 (2xs, 2x Ph); 8.01 and 8.29 (2xs, H_2 and H_8).
IR: 3410 (m), 1625 (s), 1570 (m).
Acc mass: Calc. for $C_{36}H_{54}N_5O_7PSi_2$ + H :756.33791; found $[M + H]^+$: 756.33832.

Dibenzyl ester of N^6-benzylmalonyl-2',3'di(*t*-butyldimethylsilyl)-adenosine 5'phosphate (10)
Under a N_2-atmosphere 1.0 g DCC (4.9 mmol) was added to a solution of 1.01 g **8** (1.34 mmol) and 0.62 g monobenzylmalonic acid (3.17 mmol) in 5 ml. dry pyridine at 0 °C. After 30 min. at 0 °C the reaction mixture was stirred for two days at room temperature. The remaining DCC was converted to DCU with water and filtered off. The filtrate was evaporated and flash chromatography yielded 0.86 g of a colourless oil (0.92 mmol, 69%).R_f: 0.78 (EtOAc).
^{1}H-NMR : - 0.27 (s, 3H); -0.05 (s, 3H); 0.06 and 0.07 (2xs, 6H); 0.78 and 0.91 (2xs, 2x9H); 4.01 (s, CH_2); 4.34-4.14 (m, $H_{3'}$, $H_{4'}$, $H_{5'}$); 4.78 (dd, J = 4.5 Hz, $H_{2'}$); 5.02 (m, 2x CH_2Ph); 5.21 (s, CH_2Ph); 5.97 (d, J = 4.8 Hz, $H_{1'}$); 7.32 (3xs, 3x Ph); 8.22 and 8.48 (2xs, H_2 and H_8); 9.4 (bs, NH).
IR: 3385 (s), 1750-1700 (s), 1610 (s), 1590-1570 (m).
UV (ethanol): λ_{max}: 274 nm
Acc mass: Calc. for $C_{46}H_{62}N_5O_{10}PSi_2$ + Na :954.36708; found $[M + Na]^+$: 954.36761.

Dibenzyl ester of N^6-benzylmalonyl-adenosine 5'phosphate (11)
Under a N_2-atmosphere 153 mg TBAF (0.49 mmol) was added to a solution of 226.4 mg **10** (0.24 mmol) in 4 ml. dry THF at 0 °C. The reaction mixture was stirred 45 min at 0 °C and 15 min at room temperature. The mixture was partialy evaporated and immediately flash chromatoghraphed yielding 149 mg of a white foam (0.21 mmol, 87 %). R_f : 0.63 (CH_2Cl_2/MeOH 85:15).
^{1}H-NMR: 4.01 (s, CH_2); 4.38-4.14 (m, H_3·, H_4·, H_5·); 4.55 (dd, J = 4.6 Hz, H_2·); 4.86 (d, J = 8.7 Hz, CH_2Ph); 4.95 (d, J = 8.7 Hz, CH_2Ph); 5.18 (s, CH_2PH); 6.12 (d, J = 4.8 Hz, H_1·); 7.11-7.24 (m, 2x OH); 7.26 and 7.30 (2xs, 3x Ph); 8.43 and 8.50 (2xs, H_2 and H_8); 10.4 (bs, NH).
^{13}C-NMR (50 MHz,): 44.7 (OC$\underline{C}H_2$CO); 67.1 (C_5·); 69.6, 69.8 and 69.9 (3x O$\underline{C}H_2$Ph); 70.8 (C_3·); 74.8 (C_2·); 82.8 (d, J = 7.6 Hz, C_4·); 88.4 (C_1·); 121.7 (C_5); 127.6-128.8 (3x Ph); 135.0, 135.1 and 135.2 (3x Ph); 142.0 (C_8); 143.4 (C_4); 148.6 (C_2); 151.2 (C_6); 166.1 (CO); 167.2 (CO).
IR: 3600-3100 (bs), 1745 (s), 1695 (s), 1605 (s), 1580 (s), 1465 (s).
Acc mass: Calc. for $C_{34}H_{34}N_5O_{10}P$ + H :704.21230; found $[M + H]^+$: 704.21271.

Ammoniumsalt of N^6-malonyl adenosine 5' phosphate (4)
154 mg of **11** (0.22 mmol) was hydrogenated with 16 mg $Pd(OH)_2$/C (Pearlman's catalyst) in 2 ml ethanol and 2 ml NH_4CO_3-buffer (pH= 7-8) in 2 hours at room temperature and atmospheric pressure. The catalyst was filtered off over hy-flow and the filtrate lyophilized. This yielded 106 mg of a white solid (0.22 mmol, 100%). M.p. 115-118 °C (dec.)
^{1}H-NMR (d_6DMSO): 3.2 (s, CH_2); 3.9 (m, H_4·, H_5·); 4.3 (m, H_3·); 4.6 (dd, J = 4.6 Hz, H_2·); 6.0-4.5 (bs, 2x OH); 6.0 (d, J = 5.7 Hz, H_1·); 8.3 (bs, NH_4^+); 8.6 and 8.7 (2xs, H_2 and H_8).
^{13}C-NMR (50 MHz, D_2O): 65.7 (d, J = 4.0 Hz, C_5·); 72.4 (C_3·); 76.5 (C_2·); 86.3 (d, J = 8.8 Hz, C_4·); 89.4 (C_1·); 124.6 (C_5); 145.0 (C_8); 150.3 (C_4); 153.5 (C_2); 157.1 (C_6); 171.8 (CO); 176.2 (CO).
UV (ethanol): λ_{max}: 274 nm, ε= 8400 $l.mol^{-1}.l^{-1}$.
Mass spectrum (FAB): M^+ 484; 466 (M^+-18, NH_4); 448 (M^+-36, 2x NH_4); 422 (M^+-62, CO_2NH_4).

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Received 9/2/91

Accepted 4/21/92