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Research Article

Synthesis of derivatives of 4-hydroxy isoleucine from Fenugreek and evaluation of their anti diabetic activity

Sridevi P*, Bhagavan Raju M, Harikumar Verma V and Rangarao P

Department of Pharmaceutical Chemistry, CM College of Pharmacy, Maisammaguda, Dhulapally, Secunderabad-14

***Correspondence Info:**
Sridevi Pingala
Department of Pharmaceutical chemistry,
CM College of Pharmacy, Maisammaguda,
Dhulapally, Secunderabad-14, India
E-Mail: sri_pingala@yahoo.co.in

Abstract

The new method was prepared for the extraction of 4-hydroxy isoleucine from fenugreek seeds. Similarly semi-synthetic derivatives were prepared with acceptable degree of purity and confirmed by spectral analysis. The anti-diabetic activity of semi synthetic derivatives was compared with the parent 4-hydroxy isoleucine and they showed increased activity than the parent compound.
Keywords: 4-hydroxy isoleucine, fenugreek, semi-synthetic derivatives, anti-diabetic activity

1. Introduction

Fenugreek -*Trigonella foenum-graecum* is an annual plant in the family Fabaceae. The plant has small round leaves, is cultivated worldwide as a semi-arid crop, and is a common ingredient in dishes from the Indian subcontinent. It is one of the oldest medicinal plants and is native to southeastern Europe, Northern Africa and Western Asia, but it is widely cultivated in other parts of the world. It is known commonly fenugreek. Fenugreek seeds often have a pungent aroma and may have a bitter taste, which is said to be similar to celery. The seeds of the plant contain many active compounds such as iron, vitamin A, B, C, phosphates, flavonoids, saponins, alkaloids such as trigonelline and amino acids.

1.2 4-Hydroxy isoleucine: 4 - Hydroxyisoleucine is extracted from the Fenugreek seeds which are having characteristic smell and bitter taste that of fenugreek seeds and leaves. The extracted natural active chemical is highly hygroscopic .The extract has several constituents. The active compound 4-HIL is isolated by several purifications .The other constituents steroidal saponins (diosgenin, yamogenin, tigogenin, and neotigogenin) is also isolated separately from the extracts and mucilaginous fiber which are believed to be responsible for many of the beneficial effects fenugreek is isolated separately.

4-Hydroxyisoleucine is a branch amino acid. The studies have confirmed the presence of 4 hydroxy isoleucine in fenugreek seeds in two diastereo isomers: the major one being the (2S, 3R, and 4S) configuration, representing about 90% of the total content of 4-hydroxyisoleucine, and the minor one being the (2R, 3R, 4S) configuration. The major isomer is presently interesting with respect to experimental evidence indicating its ability to stimulate glucose-induced insulin secretion in micro molar concentrations. Some studies have also shown that the natural analogue of 4-hydroxyisoleucine is more effective as an anti diabetic agent than a synthetic version.[3, 4]

Structure of 4-Hydroxy isoleucine

1.3 Biological uses

- 4-Hydroxy isoleucine in fenugreek extract plays a valuable role in insulin promotion and glucose regulation which may help to reduce body fat. [13]
- It has nutrient partitioning effect, which means it has help to shuttle nutrients to muscle cells preferentially over fat cells.[7]
- It appears to be glucose dependant. The higher is the blood glucose level is, the greater the insulin promoting response elicited by 4- hydroxyl isoleucine. By helping to regulate insulin needs, 4-hydroxyisoleucine works as an adaptogen. It is a non-protein amino acids isolated from fenugreek seeds.[10]
- 4 - hydroxy-isoleucine will be more efficient as creatine in the muscle cells to enhance muscle strength and lean muscle mass and increase strength and size of muscle cells.[6]

- It has been used to treat bronchitis and asthma. It is also considered a good herbal remedy for sore throat and coughs. It has been used to promote hair growth both in women and men[9].
- 4-hydroxyisoleucine (an amino acid derived from fenugreek) may help stimulate the secretion of insulin, reduce insulin resistance, and decrease blood sugar levels in diabetes patients, according to a 2009 study on mice[11].
- Insulinotrophic and anti diabetic properties also have been associated with the amino acid 4-hydroxyisoleucine that occurs in fenugreek at a concentration of about 0.55%. In vitro studies have indicated that this amino acid causes direct pancreatic β-cell stimulation[9]

2. Materials and Methods

The IR spectra are recorded by using Schimadzu Perkin Edmer 8201 PC IR Spectrometer using a thin film on KBR pellets technique and frequencies were expressed in cm-[1]. The PMR spectra were recorded on Bruker Avance II 400NMR spectromet [13] All spectra were obtained in $CDCl_3$ and chemical shift values are reported as values in pm relative to TMS (d=0) as internal standard.

2.1 Experimental: Milled and powdered seeds of the plant (800 g) were macerated using petroleum ether(2 l, 24 h, 4 times) and ethanol 50% (1 l, 24 h,4 times), respectively. In order to determine the free amino acids in each extract, spot test was performed using cellulose paper and ninhydrin as reagent. The presence of amino acids was confirmed by purple spots on the paper. The results were obtained from spot test showed the presence of amino acids only in ethanol extract (total volume: 4 l). In order to separation of amino acids from ethanol extract, ion exchange chromatography method was used. At first, the column (2.5 × 40 cm) was washed with distilled water (0.5 L) and HCl 2 N (1 L), respectively, for 3 days. After acidifying the 225H cationic resin, distilled water was passed from the column until pH of elute became neutral. Then 100ml ethanol extract was subjected to the cationic exchange column and eluted with distilled water (1 L) and ammonium hydroxide 1N (1 L), respectively.

The fractions were collected (100 ml each one) when pH of elute became alkaline. Spot test was performed on each fraction and the fractions containing amino acids were mixed together (fractionA)[13].

2.2 Preparation of semi synthetic derivatives: Synthesis of 2-Amino-4-hydroxy-3-methyl Pentatonic acid methyl ester (MI), Synthesis of 2-Amino-4-hydroxy-3-methyl Pentanoic acid ethyl ester (EII), Synthesis of 2-Amino-4-hydroxy-3-methyl Pentanoic acid Benzyl ester (BIII), Synthesis of 2-Amino-4-hydroxy-3-methyl Pentanoic acid -4-nitro Benzyl ester (BIV), Synthesis of 2-Amino-4-hydroxy-3-methyl Pentanoic acid -2-Nitro Benzyl ester (BV), Synthesis of 2-Amino-4-hydroxy-3-methyl Pentanoic acid 2,4 dinitro Benzyl ester (BVI), Synthesis of 2-Amino-4-hydroxy-3-methyl Pentanoic acid butyl ester (BVII).

2.3 Procedure: 4-Hydroxy isoleucine (10mmol) was taken in a beaker. 100ml of dimethoxy propane and 10ml of hydrochloric acid (MI)/50ml of ethanol and 3.8gm of Para – toluene sulphonic acid (EII)/ 48.5gm of Para-toluene sulphonic acid was added to a mixture of 100ml benzyl alcohol and 50ml benzene (BIII)/ 100ml p-nitrobenzyl alcohol and 50ml benzene (BIV)/ 100ml of 2-nitro benzyl alcohol and 50ml benzene (BV)/ 100ml of 2,4 di-nitro benzyl alcohol and 50ml benzene (BVI)/ 50ml of butanol and 3.8gm of Para – toluene sulphonic acid (BVII) was added. Stirred for 12 hrs at 60°c. Filter off the compound and the residue is dissolved in methanol and diluted with dry ether Filter the above product and it was recrystallized with ether and methanol.[14, 15]

Scheme:

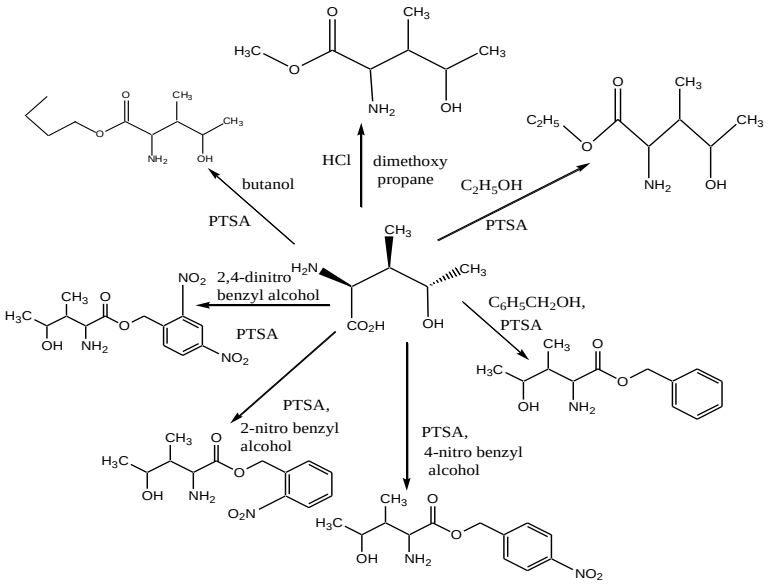


Table 1. Physical data

S. No	Compound	Physical state	Yield (%)	R_f (N-butanol:Acetic acid:water)
1	MI	Solid	76	0.5
2	EII	Solid	81	0.6
3	BIII	Solid	68	0.4
4	BIV	Semi-solid	74	0.5
5	BV	Semi-solid	78	0.8
6	BVI	Semi-solid	67	0.6
7	BVII	Semi-solid	65	0.7

2.4 Evaluation of biological activity

2.4.1 Animals: Swiss Albino mice of either sex (25-30g) were obtained from Damoder chemicals, Hyderabad. Animals were housed under standard condition of temperature (25 ± 2°C), 12h/12h light dark cycles and fed with standard pelleted diet and water was given *ad libitum.* Animal handling was performed as per *Good Laboratory Practice*. A research proposal was prepared according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

2.4.2 Drugs: The solution of synthetic derivatives from 4HIL were prepared in distilled water and administered in the doses of 30, 60 and 120 mg/kg, p.o. appropriate dilutions were made for preparation of the suitable dose so as to administer *per orally* 0.1 ml / 10 g of body weight of mice

2.4.3 Acute study: Animals were fasted overnight before commencing the Experiment. Blood glucose was estimated before giving any drug and the readings were noted as 0 h reading. Drugs were administered to respective groups orally and blood glucose was estimated at 2h, 4h, 6h and 24h for acute study (VII).

2.4.4 Sub acute study: The drugs were administered daily for 28 days at a prefixed time Blood glucose was estimated on weekly intervals. At the end of 28 days the drug administration was stopped and a rest period

Of 7 days was given to the animals to study effect of drug treatment on blood glucose after 7 days.

2.4.5 Histology of pancreas: After seven days rest period, the animals were sacrificed and pancreas was isolated to study regenerative ability of pancreas. Histological examinations were carried out using Haematoxylin.[1]

2.4.6 Determination of LD_{50}: Acute oral toxicity and LD50 determination were carried out.

2.4.7 Induction of diabetes and estimation of blood glucose: Alloxan monohydrate (70 mg/kg, i.v) was used to induce diabetes in mice. Blood was withdrawn by retro orbital plexus technique and blood glucose was estimated by glucose oxidase peroxides (GOD/POD) method using kit obtained from Accurex Biomedical, Normal mice were made diabetic by giving them alloxan monohydrate (70 mg/kg, i.v). After 48 hours, the blood was withdrawn and blood glucose was estimated. The animals showing blood glucose more than 200 mg/dl were called diabetic' and were selected for the study (VI). The animals were divided into four groups (diabetic) and one group of non diabetic animals each containing 6 animals. Group I - Control (non diabetic), Group II - Only alloxan (70 mg/kg, i.v), Group III – Only glyburide (10 mg/kg), Group IV– MI, EII, BIII, BIV, BV, BVI&BVII. The synthetic derivatives were administered at three dose levels (30, 60 and 120 mg/kg). In the present study, the dose of 120 mg/kg was selected because lower dose i.e. 30 and 60 mg/kg did not decrease the blood glucose significantly.

2.4.8 Determination of glycosylated hemoglobin (HbA1c): At the end of the study, the blood samples were taken from the animals. Glycosylated hemoglobin was estimated by using the chromatographic spectrophotometric ion exchange method using the kit obtained from Biosource.[8]

2.4.9 Intraperitoneal glucose tolerance test (IPGTT) in diabetic mice: Diabetic animals were fasted overnight and blood glucose was determined before commencing the experiment, which was considered as normal. Drug was administered to the animals and half an hour later glucose (2.5g/kg) was administered intraperitoneally to the animals. Blood glucose was estimated immediately after administration of glucose which was considered as 0 min reading and further at 30, 60 and 120 min

2.4.10 Body weight and mortality: During the study period of 35 days, animals were weighed daily and their body weight was recorded. Death of animals was noted and percentage mortality was calculated.

2.4.11 Statistical analysis: Data was expressed as mean ± S.E.M and statistical analysis was carried out by One Way ANOVA followed by post-hoc Tukey's test using Graph Pad InStat version 3.00 for Windows 95, Graph Pad Software, San Diego California USA.

3. Results

Table 2. Result of spectral analysis

S. No	Compound	IR(cm^{-1})	NMR(400MHz,$CDCl_3$) τ Values	MASS(m/e)
1	MI	N-H str-3170, $COOCH_3$-1735, OH str-3235.	$COOCH_3$(s8.2)CH_3(s3.67),CH(m3.44-2.6), CH_3(m1.21-1.06), NH_2 (t 2.08), OH (t 2.1)	147
2	EII	N-H str-3170 $COOCH_3$-1735 OH str-3235	$COOC_2H_5$(s8.2)$COOCH_2$(d4.12), $COOCH_2CH_3$(t1.30), CH(m3.44-2.6), CH_3(m1.21-1.06), NH_2 (t 2.08), OH (t 2.1)	162
3	BIII	N-H str-3050 $COOC_6H_5$-1708 OH-3429C-O str-1240C=C(aromatic)-1470	$COOC_6H_5$(s8.4)$COOCH_2$ (m 5.38), C_6H_5(m7.19),CH(m3.44-2.6), CH_3(m1.21-1.06), NH_2 (t 2.08), OH (t 2.1)	224
4	BIV	N-H str-3050 $COOC_6H_5$-1708 OH-3429 C-O str-1240 C=C(aromatic)-1470, Ar NO_2-1392	$COOCH_2$(m5.34), $C_6H_4NO_2$(m7.45), CH(m3.44-2.6), CH_3(m1.21-1.06), NH_2 (t 2.08), OH (t 2.1)	285
5	BV	N-H str-3050 $COOC_6H_5$-1708 OH-3429 C-O str-1240 C=C(aromatic)-1470, Ar NO_2-1392	$C_6H_4NO_2$(m7.45), $COOCH_2$(m5.43),CH(m3.44-2.6), CH_3(m1.21-1.06), NH_2 (t 2.08), OH (t 2.1)	285
6	BVI	N-H str-3050 $COOC_6H_5$-1708 OH-3429 C-O str-1240 C=C(aromatic)-1470, Ar NO_2-1392	$C_6H_3(NO_2)_2$(m9.05), $COOCH_2$(m5.37),CH(m3.44-2.6), CH_3(m1.21-1.06), NH_2 (t 2.08), OH (t 2.1)	347
7	BVII	N-H str-3170 $COOC_4H_4$-1735 OH str-3235	$COOCH_2$(t4.08), $COOC_4H_4$(s8.4) C_3H_7(m0.96-1.57), CH(m3.44-2.6), CH_3(m1.21-1.06), NH_2 (t 2.08), OH (t 2.1)	213

3.1. Evaluation of Biological Activity

3.1.1 Acute study

The onset of blood glucose decrease in synthetic derivatives (120 mg/kg) was seen at 2h. The peak decrease in blood glucose appeared to be at 6h in case of (127.05 and 279.45 mg/dl). 4HI showed more reduction in blood glucose than methyl treated group at all hours. Values are mean ± S.E.M, n=6 in each group; Statistical analysis by one – way ANOVA followed by Tukey's test using Graph pad In stat software; P values < 0.01a, P < 0.001b compared to only alloxan group and P<0.01c, P<0.001d as compared to MI,EII,BIII,BIV,BV,BVIAND BVII. Drugs were administered orally.

3.1.2 Sub acute study

Results indicated gradual significant decrease in blood glucose on continued administration of semi synthetic derivatives. Maximum decrease in blood glucose was observed after rest period of fifteen days in the animals treated with semi synthetic derivatives respectively. 4 HIL and some semi synthetic derivatives showed significant decrease in blood glucose as compared to glyburide group for 5th, 10th and 15th day. These results indicated that maximum reduction occurred after fifteen days of rest period may be due to the action.

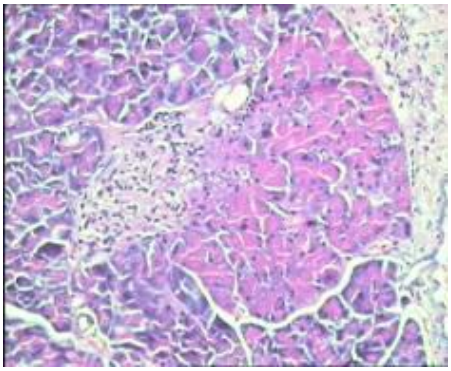
Table 3. Blood Glucose level

	Initial Day	Day5	Day10	Day15
Normal	72.25 ±0.8	73.5±1.0	73.75±0.86	75.5±1.58
Diabetic control	185±2.88	186.2±1.7	189.5±1.25	192.5±1.73
MI	192±2.6	151±1.5	110.6±3.0*	80.6±1.8**
EII	190.3±4.98	148±1.1**	108±3.4*	77.6±1.4**
BIII	197.3±4.3	157.6±5.2**	129.6±3.2*	89.3±2.5**
BIV	194 ± 2.8	148.3 ± 3.1	116.6 ± 5.3*	89.3 ± 2.5**
BV	185.3 ± 4.2	154 ± 1.1	134.6 ± 2.4	88.6±1.2**
BVI	186.3 ± 4.3	146.3 ± 5.3*	121.3 ± 1.4	80.3 ± 0.9
BVII	190.3 ± 4.98	148 ± 1.1**	108 ± 3.4*	84.6 ± 1.8**

Data represent means +_ S. E. M, P*<0.05, P**<0.0001

In 4HI treated animals (Group IV) pancreatic section had many islets occasionally around the duct.

Fig 1. Pancreatic section of MI,EII,BIII,BIV,BV,BVI&BVII showing many islets occasionally around the duct indicating Pancreatic regeneration X 200.



3.1.3 Mortality of animals

Administration of alloxan resulted in death of 57 % of the total animals during 28 days study period. Administration of both glyburide and synthetic derivatives showed reduced mortality to 45% and 35% respectively. Administration of semi synthetic derivatives reduced mortality to 35%. It was thus apparent from the results that when no drug was administered progression of diabetes resulted in mortality of mice, while the anti diabetic effect of semi synthetic derivatives prevented mortality.

3.1.4 Determination of LD^{50}: LD50 determination was done as per OECD guidelines using AOT425 software. LD50 of 4HIL, glyburide and semi synthetic derivatives was found to be greater than 5000mg/kg.

3.1.5 Hypoglycemic activity[1,2]: The aim of the study was to evaluate the hypoglycemic activity of synthetic compounds from 4 hydroxyisoleucine in alloxan induced diabetic mice. The compounds were isolated by column chromatography from fenugreek seeds. The compounds were administered orally in alloxan induced diabetic mice. The parameters studied were blood glucose, histology of pancreas, body weight, mortality and acute oral toxicity. 4HI (120 mg/kg) and synthetic derivatives showed reduction in blood glucose level within 2h and reduced the peak blood glucose level at 6h during acute study. After 28 days treatment with 4HI, there was significant decrease in blood glucose level. Synthetic derivatives increased the glucose threshold as compared to only alloxan treated group. Histology of pancreas showed formation of new islets near the vicinity of the pancreatic duct. Decreased glycosylated hemoglobin adds to the effect of 4HI. Glyburide was used as a standard anti diabetic drug and its effect on pancreatic cell was also studied. The pancreatic beta cells of glyburide treated mice did not show any islets in the vicinity of pancreatic duct.Both synthetic derivatives and glyburide arrested the decrease in body weight and mortality of diabetic mice. LD50 was found to be more than 5000 mg/kg.[10] these results suggest that synthetic derivatives from 4HIL showed hypoglycemic effect in alloxan induced diabetic mice. The presence of the pancreatic islets in the vicinity of duct suggested synthetic derivatives might act by regeneration of new islets.

4. Conclusion

4-Hydroxy isoleucine is extremely interesting compound and exerts various biological activities. The purity of 4-Hydroxy isoleucine was increased about 90% than earlier method and it is considered as one of "the greatest drug with potential growth in coming centuries". Its semi synthetic derivatives are confirmed by IR, MASS and NMR. They showed improved biological activities than parent compound .Anti diabetic activity of semi synthetic compounds like MI, EII, and BVII showed greater anti diabetic activity.

References

1. Cerasi E., Luft R., Efendic S. Decreased sensitivity of the pancreatic beta-cells to glucose in prediabetic and diabetic subjects: a glucose dose response study. *Diabetes* 1972: 224–234.
2. Fajans S. S., Floyd J. C., Knopf R. F., Guntsche E. M., Rull J. A., Thiffault C. A.,Conn J. W. A difference in mechanism by which leucine and other amino acids induce insulin release. *J. Clin. Endocrinol. Metab*. 1967; 1600–1606.
3. Raghuram T.C., Sharma RD., Sivakumar B., Sahay B. K. Effect of fenugreek seeds on intravenous glucose disposition in non-insulin dependent diabetic patients. *Phyto. Res.* 1994:83–86.
4. Sauvaire Y., Petit P., Broca C., Manteghetti M., Baissac Y., Fernandez AlvarezJ., 4-hydroxyisoleucine:a novel amino acid potentiator of insulin secretion. *Diabetes* 1998: 206–210.
5. Trinder P. Determination of blood glucose using an oxidase peroxidase system with a non-carcinogenic chromagen. *J. Clin. Pathol*. 1969158–161.
6. Anthony JC**,** Anthony TG, Kimball SR, and Jefferson LS. Signaling pathways involved in translational control of protein synthesis in skeletal muscle by leucine. *J Nutr* 2001: 856S–860S.
7. Gao Z**,** Konrad RJ, Collins H, Matschinsky FM, Rothenberg PL, and Wolf BA**.** Wortmannin inhibits insulin secretion in pancreatic islets and beta-TC3 cells independent of its inhibition of phosphatidylinositol3kinase. *Diabetes*1996: 854–862.
8. Kahn CR**.** Insulin receptors and insulin signaling in normal and disease states. In: *International Textbook of Diabetes Mellitus* (2nd ed.), edited by Alberti KGMM, Zimmet P, DeFronzo RA, and Keen H. Philadelphia, PA: Lippincott-Raven, 1997, p. 437–467.
9. Rohner-Jeanrenaud F**,** Proietto J, Ionescu E, and Jeanrenaud B. Mechanism of abnormal oral glucose tolerance of genetically obese rats. *Diabetes* 1986; 35:1350–1355.
10. Shani J, Goldschmidt A, Joseph B, Aaronson Z, Selman FG: Hypoglycemic effect of *Trigonella forenoon guaiacum* and *Lupines' term's* (Leguminosae) seeds and their major alkaloids in alloxan-diabetic and normal rats. *Arch In Pharmacodyn There* 1974; 210:27-36
11. Gupta RK, Kasseri AN, Watal G, *et al*. Hypoglycemic andantidiabetic effect of aqueous extract of leaves of *Annona squamosa* (L.) in experimental animal. *Cur. Sci*. 2005; 88:1244-1254.
12. Sauvaire Y, Petit P, Broca C, Manteghetti M, Baissac Y, Fernandez-Alvarez J 4-Hydroxyisoleucine: a novel amino acid potentiator of insulin secretion. *Diabetes.* 1998 Feb; 47(2):206-10.
13. Valérie Rolland-Fulcrand, Marc Rolland, Marie-Louise Roumestant, Jean Martinez Chemoenzymatic Synthesis of Enantiomerically Pure (2*S*,3*R*,4*S*)-4-Hydroxyisoleucine, an Insulinotropic Amino Acid Isolated from Fenugreek Seeds. *European Journal of Organic Chemistry* 2004; (4): 873–877.
14. Jing Xiong, Hai-Feng Zhu, Ya-Juan Zhao, Yun-Jun Lan, Ji-Wang Jiang, Jing-Jing Yang and Shu-Feng Zhang *:* Synthesis and Antitumor Activity of Amino Acid Ester Derivatives Containing 5-Fluorouraci**l** *Molecules* 2009, *14*(9), 3142-3152;
15. Roman Mazurkiewicz, Anna Kuźnik, Mirosława Grymel,and Agnieszka Październiok-Holewa α-Amino acid derivatives with a Cα-P bond in organic synthesis. *ARKIVOC* 2007 (vi) 193-216.